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TITLE: DETERMINATION OF ACTIVITY PARAMETERS IN THIOBACILLUS FERROOXIDANS STRAINS AS CRITERIA FOR MINERAL LEACHING EFFICIENCY (UP-M-589)

FINAL REPORT

BY

Isamu Suzuki
Department of Microbiology
The University of Manitoba

(Coworkers)

Jae Key Oh, Patrick D. Tackaberry,
Hector M. Lizama, Travis L. Takeuchi

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GENERAL EXECUTIVE SUMMARY

We have grown three *Thiobacillus thiooxidans* strains (a laboratory strain, Tt = ATCC 8085; mine isolates, SM-6 and SM-7) on elemental sulfur (S^0) and eight *Thiobacillus ferrooxidans* strains (laboratory strains, Tf-1 = ATCC 13661 and Tf-2 = ATCC 19859; mine isolates, SM-1, SM-2, SM-3, SM-4, SM-5 and SM-8) on ferrous iron (Fe^{2+}) or elemental sulfur (except SM-2) as sole energy source and studied various activity parameters as possible criteria for mineral leaching efficiency.

Growth on S^0 or Fe^{2+}, metal tolerance (Cu, Zn, Ag, Hg, Mo, As), adaptation to metals, S^0 or Fe^{2+} oxidation activity, effect of metals on the oxidation, and mineral leaching activity were studied. SM-4 had the highest score points among Fe^{2+}-grown cells and SM-6 and SM-7 among the S^0-grown cells. All three were also efficient in Cu and Zn leaching from a sulfide ore and would be the best strains to be used for other ores. SM-3 performed well in most parameters, but failed to leach metals in most experiments. SM-8 and Tf-2 scored high and their sulfur-grown cells leached Cu and Zn better than their Fe^{2+}-grown cells. They would be good strains to be tested on sulfide ores perhaps after adaptation. Tf-1 was mediocre in performance and poor in metal leaching. SM-5, an overall mediocre strain, performed better when it was grown on Fe^{2+} than on S^0 and only the Fe^{2+}-grown culture leached Cu and Zn from ore. SM-1 was a poor performer in general. It grew well on S^0, but failed to leach metals from ore. SM-2 failed to grow on S^0 and had the lowest total score of all strains studied.

It is concluded that these parameters studied are useful in selecting the best strains for leaching metals from a sulfide ore, but additional parameters such as the ability to adsorb on solid surfaces and the ability to oxidize sulfur with ferric iron should also be studied for a more complete evaluation of strains. We are currently investigating these properties.

RÉSUMÉ EXÉCUTIF GÉNÉRAL

Nous avons cultivé trois souches de *Thiobacillus thiooxidans* (une souche de laboratoire, Tt = ATCC 8085; des isolés de mines, SM-6 et SM-7) sur du soufre élémentaire (S^0) et huit souches de *Thiobacillus ferrooxidans* (des souches de laboratoire, Tf-1 = ATCC 13661 et Tf-2 = ATCC 19859; des isolés de mine, SM-1, SM-2, SM-3, SM-4, SM-5 et SM-8) sur du fer ferreux (Fe^{2+}) ou du soufre élémentaire (sauf SM-2) comme source unique d'énergie et nous avons étudié les paramètres d'activité divers comme critères possibles pour l'éfficacité de lixiviation du minerai.

La culture sur S^0 ou Fe^{2+}, la tolérance métallique (Cu, Zn, Ag, Hg, Mo, As), l'adaptation aux métaux, l'activité d'oxydation, et l'activité de lixiviation minerale ont été étudiés. SM-4 avait le plus haut score entre les cellules cultivées avec Fe^{2+} et SM-6 et SM-7 parmi les cellules cultivées avec le S^0. Toute les trois étaient aussi éfficaces dans la lixiviation de Cu et Zn d'un minerai sulfuré et seraient les meilleures souches pour être utilisées avec d'autres minerais. SM-3 a bien opèré dans la plupart des paramètres mais n'a pas réussi à lixivier les métaux dans la plupart des expériences. SM-8 et Tf-2 ont compté haut et leurs cellules cultivées dans le soufre ont lixivié le Cu et le Zn mieux que leurs cellules cultivées dans le fer ferreux. Ils seraient de bonnes souches pour être testées sur des minerais sulfurés, peut-être après une adaptation. Tf-1 était médiocre dans sa performance et pauvre dans la lixiviation des métaux. SM-5, une souche pour la plus part médiocre, a mieux opéré, quand elle a été cultivée sur Fe^{2+} que sur S^0 et seulement la culture-Fe^{2+} a lixivié Cu et Zn dn minerai. SM-1 a donné un rendement pauvre général. La croissance était bonne sur le S^0, mais cette souche n'a pas réussi à lixivier les métaux du minerai. SM-2 n'a pas poussé sur S^0 et avait le plus

bas score de toutes les souches étudiées.

Il est conclu que ces paramètres étudiés sont pratiques pour la sélection des meilleures souches pour la lixiviation des métaux de minerai sulfuré, mais des paramètres additionnel, par exemple, la capacité d'adsorber sur des surfaces solides, et la capacité d'oxydation du soufre avec le fer ferreux devraient aussi être étudiées pour une évaluation plus complète des souches. Nous sommes présentement en train d'investiguer ces propriétés.

INTRODUCTION

Bacterial leaching of sulfide ores using *Thiobacillus ferrooxidans* has been an important process for the extraction of economically valuable metals from ores not amenable to traditional methods and has an advantage of not releasing the oxidized sulfur (e.g. SO_2) into the atmosphere.

T. ferrooxidans is able to grow either on ferrous iron or inorganic sulfur compounds and use the energy released during the oxidation ($4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$ or $S^0 + 1\ 1/2\ O_2 + H_2O \rightarrow H_2SO_4$). *Thiobacillus thiooxidans*, a closely related bacterium specializes in the oxidation of sulfur. Selected strains of both organisms can leach metals from various sulfide ores.

In spite of its great potential the use of bacterial leaching has been limited mainly because of the relatively slow rate and low concentration of metals extracted into solution. The rate is presumably related to the growth of bacteria and oxidation of sulfur or sulfide and ferrous iron by the bacterial cells. The concentration of metals extracted is presumably limited by the inhibition of bacterial growth and oxidation by the metals released during the leaching.

We have therefore studied a number of *T. ferrooxidans* and *Thiobacillus thiooxidans* strains on various parameters relating to growth and activity including the effects of metals (Cu, Zn and other inhibitory metals often released from sulfide ores) in an effort to establish criteria essential for the selection of strains best suited for the effective leaching of metals from a sulfide ore.

STAGE I

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EXECUTIVE SUMMARY

We have grown three Thiobacillus thiooxidans strains (Tt = ATCC 8085, SM-6 and SM-7) and seven Thiobacillus ferrooxidans strains (Tf-1 = ATCC 13661, Tf-2 = ATCC 19859, SM-1, SM-3, SM-4, SM-5, and SM-8) on elemental sulfur and eight T. ferrooxidans strains (Tf-1, Tf-2, SM-1, SM-2, SM-3, SM-4 SM-5 and SM-8) on ferrous iron as the sole energy source. Cu and Zn tolerance of these strains was studied both during growth and with resting cell suspensions.

During growth on sulfur SM-4, Tt, SM-3, SM-6 and SM-7 were tolerant of 50 mM $CuSO_4$; SM-7, SM-6 and SM-3 were tolerant of 200 mM $ZnSO_4$; and all the strains were tolerant of 200 mM $FeSO_4$.

The sulfur-oxidizing activity of SM-3, SM-4, SM-6 and SM-7 was resistant to inhibition by 167 mM $CuSO_4$ and 417 mM $ZnSO_4$.

Growth on Fe^{2+} was rapid with Tf-1, Tf-2, SM-3 and SM-4 with complete oxidation of Fe^{2+} in 25 hours, but SM-1, SM-2, SM-5 and SM-8 were slow growers.

$CuSO_4$ at 10 mM had no effect, but at 30-100 mM inhibited the growth of all strains by increasing the length of lag period after which they all grew although more slowly.

All the strains tested grew at 100 or 150 mM $ZnSO_4$ with only minor inhibition.

The Fe^{2+}-oxidizing activity of all strains was inhibited by Fe^{3+}, Cu^{2+} and Zn^{2+}, generally competitively with respect to Fe^{2+} concentration.

In conclusion the fresh isolates were generally more tolerant of metals when grown on sulfur than laboratory strains which were maintained in laboratory media for a long period of time.

RÉSUMÉ EXÉCUTIF

Nous avons cultivé trois souches Thiobacillus thiooxidans (Tt = ATCC 8085, SM-6 et SM-7) et sept souches Thiobacillus ferrooxidans (Tf-1 = ATCC 13661, Tf-2 = ATCC 19859, SM-1, SM-3, SM-4, SM-5 et SM-8) sur du soufre élémentaire et huit souches T. ferrooxidans (Tf-1, Tf-2, SM-1, SM-2, SM-3, SM-4, SM-5 et SM-8) sur de fer ferreux comme source unique d'énergie. La tolérance en Cu et en Zn de ces souches a été étudiée aussi bien durant la croissance qu'avec des cellules de suspension au repos.

Au cours de la croissance sur soufre, SM-4, Tt, SM-3, SM-6 et SM-7 avaient une tolérance de 50 mM $CuSO_4$; SM-7, SM-6 et SM-3 toléraient 200 mM $ZnSO_4$; toutes les souches avaient une tolérance de 200 mM $FeSO_4$. L'activité oxydante du soufre de SM-3, SM-4, SM-6 et SM-7 résistait a l'inhibition de 167 mM $CuSO_4$ et de 417 mM $ZnSO_4$.

La croissance sur Fe^{2+} était rapide avec Tf-1, Tf-2, SM-3 et SM-4 avec oxydation complète de Fe^{2+} en 25 heures, mais SM-1, SM-2, SM-5 et SM-8 ne poussaient pas très rapidement.

À 10 mM $CuSO_4$ n'a aucun effect, mais à 30-100 mM il inhibe la croissance de toutes les souches en augmentant les temps de latence, après quoi elles croissent toutes, bien que plus lentement.

Toutes les souches qui ont été testées ont poussé à 100 ou 150 mM $ZnSO_4$ avec une seule inhibition mineure.

L'oxydation Fe^{2+} de toutes les souches a été inhibée par Fe^{3+}, Cu^{2+} et Zn^{2+}, généralement en compétition en ce qui concerne la concentration en Fe^{2+}.

En conclusion les souches nouvellement isolées étaient d'une façon générale plus tolérants des métaux lorqu'ils sont cultivés sur du soufre que les souches de laboratoirs qui ont été gardées dans un laboratoire média pendant longtemps.

MATERIALS AND METHODS

Bacterial strains

Thiobacillus thiooxidans ATCC 8085 (Tt), Thiobacillus ferrooxidans ATCC 13661 (Tf-1) and T. ferrooxidans ATCC 19859 (Tf-2) were the laboratory control strains used. SM-1, SM-2, SM-3, SM-4, SM-5, SM-6, SM-7 and SM-8 were isolated from water samples from various seepage and drainage locations at the Flin Flon Mine of the Hudson Bay Mining and Smelting Co., Ltd., Manitoba. Tt, SM-6 and SM-7 grew only on sulfur as the energy source (T. thiooxidans) and was maintained in the Starkey's No. 1 medium (1). Tf-1, Tf-2, SM-1 to 5 and SM-8 grew on Fe^{2+} as the sole energy (T. ferrooxidans) and were maintained in a modified 9K (M9K) medium (2). T. ferrooxidans strains adapted to sulfur were maintained also in the Starkey's No. 1 medium. All stock cultures were kept at 4°C.

Media and growth of bacteria on sulfur

The medium used for the growth on elemental sulfur was the Starkey's No. 1 medium (1): 0.3 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2$, and 18 mg $FeSO_4 \cdot 7H_2O$ per L of distilled water; adjusted to pH 2.3 with H_2SO_4. Powdered precipitated sulfur (BDH Chemicals) (50 g/L) was spread evenly on the surface after inoculation (2.5% v/v) as described (3). Normally a culture was grown in one liter volume in a 2.8 L Fernbach flask at 25°C without agitation, but for some experiments it was grown in 200 mL volume in a 500 mL Erlenmeyer flask or at 28°C.

Cells were collected after 7 days by removing the remaining sulfur by filtration through a Whatman No. 1 filter paper under suction, by centrifuging the filtrate and washing the cells once in the Starkey's medium. The washed cells (20-50 mg wet cells/mL in the Starkey's medium) were used within two days for the determination of the sulfur-oxidizing activity.

For the study of heavy metal effect various concentrations of heavy metal

solutions were adjusted to pH 2.3 with H_2SO_4 or NaOH and after sterilization added to the cooled sterile medium to avoid the precipitation with phosphate.

Growth rate on sulfur

Ten mL samples were removed periodically from cultures and bacterial cells were separated from elemental sulfur by filtration through a Whatman No. 1 filter and were harvested by centrifugation. The cells were washed three times with the Starkey's No. 1 medium (pH 2.3) and then were boiled for 15 min in 0.1 N NaOH for the determination of total protein concentrations by the methods of Lowry et al (4) using crystalline bovine serum albumin as a standard. Growth curves were plotted on a semi-log scale to obtain the generation time.

Determination of sulfur-oxidizing activity

The rate of sulfur oxidation by various strains was determined by measuring the rate of oxygen disappearance in an Oxygraph with a Clark electrode at 25°C. The standard reaction mixture contained 50 µL of a bacterial cell suspension (50 mg wet cells/mL), 0.3 mL sulfur (320 mg precipitated sulfur, low Fe, BDH/mL 500 ppm Tween 80) and Starkey's medium No. 1 (pH 2.3) in a total volume of 1.2 mL. The sulfur-oxidizing activity was expressed as nmol O_2/min. The effect of heavy metals on the activity was studied by including in the reaction mixture 0.1 or 0.2 mL of 1 M $CuSO_4 \cdot 5H_2O$ or 2.5 M $ZnSO_4 \cdot 7H_2O$ at pH 2.3.

Media and growth of bacteria on iron

The medium used for the growth on ferrous iron was a modified 9K (M9K) medium (2): 0.4 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, and 33.3 g $FeSO_4 \cdot 7H_2O$ per liter and adjusted to pH 2.3 with H_2SO_4. The ferrous sulfate solution was filter sterilized separately. Normally a culture was grown in 100 mL volume (10% inoculum) in a 250 mL Erlenmeyer flask at 25°C on a rotary shaker at 150 rpm, but for the purpose of obtaining cells for activity determination larger volumes and larger flasks were used. Cells were collected

by centrifugation (12,000 x g for 10 min) after removal of ferric iron by decantation or low speed centrifugation (100 x g for 10 min).

Growth rate on iron

Stock cultures of various strains kept at 4°C were subcultured three times (each subculture at 75-80% consumption of ferrous iron) before the growth curve study of actively growing cultures was carried out.

Cell growth was followed by the determination of Fe^{3+} by the yellow color (5) in a Klett-Summerson Photoelectric Colorimeter with a blue (No. 42) filter after 0.5 mL culture sample was mixed with 4.5 mL 1 N HCl. Cell growth was also followed by the protein determination (4) to confirm that the amount of Fe^{3+} ion produced was proportional to the amount of protein in the culture during the growth. The protein determination was more difficult and was affected by the presence of ferric iron towards the end of growth phase. Therefore the simple Fe^{3+} determination was used throughout the growth study, except when cells were collected the protein concentration of cell suspensions was determined. The growth curves were plotted on a semi-log scale to obtain the generation time.

Determination of Fe^{2+}-oxidizing activity

The rate of ferrous iron oxidation by various strains was determined by measuring the rate of oxygen consumption in an Oxygraph at 25°C. The standard reaction mixture contained 25 µL of a bacterial suspension (50 mg wet cells/mL), microliter volumes of 0.1 M $FeSO_4 \cdot 7H_2O$ (pH 2.0) and M9K medium (pH 2.3) in a total volume of 1.2 mL. The Fe^{2+}-oxidizing activity was expressed as nmol O_2/min. Routine assays were carried out with 10 or 50 µL of 0.1 M $FeSO_4$, but for the characterization of each strain varied volumes of the $FeSO_4$ solution were used to obtain the maximal velocity, Vmax, i.e., the activity at the saturating Fe^{2+} concentration, by the double reciprocal plots of activity

versus the Fe^{2+} concentration.

The inhibitory effect of heavy metals on Fe^{2+} iron oxidation was studied by including in the reaction mixture microliter quantities of 1 M $CuSO_4 \cdot 5H_2O$ (pH 2.3), 2.5 M $ZnSO_4 \cdot 7H_2O$ (pH 2.3) or 1 M $Fe_2(SO_4)_3$ in 0.1 M $FeSO_4 \cdot 7H_2O$ (pH 1.0) and measuring the rate of oxygen consumption at varying $FeSO_4$ concentrations. In the case of Fe^{3+} inhibition the amount of Fe^{2+} added in the inhibitor solution had to be considered. The reaction was started with the addition of bacteria. The double reciprocal plots of activity versus Fe^{2+} concentrations at different heavy metal concentrations were analyzed for the type of inhibition, apparent K_m values for Fe^{2+}, and K_i values.

RESULTS

I. Sulfur-grown cells

Adaptation to sulfur

Tt, SM-6 and SM-7 were maintained on sulfur (i.e. T. thiooxidans) and required no adaptation, but other strains were maintained on ferrous iron (i.e. T. ferrooxidans) and required adaptation periods before they grew well enough for study.

Tf-1, Tf-2, SM-3 and SM-4 adapted rapidly when transferred from the iron culture to the sulfur medium and reached the level of rapid growth equivalent to T. thiooxidans within one month (after 3-4 subcultures).

SM-1, SM-5 and SM-8 were more difficult to adapt. Initially it took them two to three weeks to reach the cell densities of one week old cultures of fully sulfur-adapted cells. Full adaptation to sulfur required 2 to 4 months of repeated transfer (3 to 4 subcultures for SM-1 and SM-8 and 6 to 7 subcultures for SM-5).

SM-2 never grew in the sulfur medium and therefore was considered incapable of using sulfur as energy source.

Characteristics of sulfur-grown cells

All strains, some after adaptation, grew rapidly on sulfur following a lag period of two to three days with a relatively uniform generation time of 7.8 to 9.5 hours and reached a cell protein concentration of 32 to 67 mg/L in 7 days at 25°C (Table 1, Figs. 1-3). As shown in Table 1 the cells collected by centrifugation had different colors characteristic of strains. The specific activity for sulfur oxidation varied four fold among different strains. Tt, Tf-1, Tf-2, SM-1, SM-6 and SM-7 cells had higher specific activities than SM-3, SM-4, SM-5 and SM-8 cells.

Effect of Cu and Zn on growth

Various strains adapted to sulfur were now grown on sulfur in the presence and absence of various heavy metals and the growth (mg protein/L) was followed for 7 days at 25°C. The effect of Cu^{2+}, Zn^{2+} and Fe^{2+} on the growth on sulfur is shown in Tables 2-4.

$CuSO_4$ had a clear-cut effect as shown in Table 2 in distinguishing Cu-tolerant strains from Cu-sensitive strains, SM-4 being the most tolerant growing to 73% of control in the presence of 50 mM $CuSO_4$ (3.2 g Cu/L) and SM-8 the least tolerant growing only to 10% of control in 10 mM $CuSO_4$ (0.6 g Cu/L). Interestingly only the Cu-tolerant strains produced black cupric sulfide and H_2S odour, probably relating to their detoxification mechanism of Cu. Some CuS was inseparably bound to SM-6 cells.

Growth inhibition by $ZnSO_4$ required higher concentrations than $CuSO_4$, but various strains were again placed in two groups, Zn-tolerant strains and Zn-sensitive strains. The former strains showed a gradation of tolerance, SM-7 and SM-6 being the most tolerant (52 and 34% growth of control at 200 mM $ZnSO_4$ or 13 g Zn/L) and SM-8 the least tolerant (Table 3). Both SM-1 and SM-5 were extremely sensitive reaching only 2-3% growth of control at 50 mM $ZnSO_4$ (3.2 g Zn). Thus SM-1 and SM-5 were very sensitive both to Cu and Zn during growth on sulfur with SM-8 being similar.

Table 4 indicates that all the strains studied were relatively tolerant of $FeSO_4$ during growth on sulfur at concentrations normally used for growth on ferrous iron (33.3 g $FeSO_4 \cdot 7H_2O$/L = 120 mM).

Growth curves for Tt, SM-6 and SM-7 with and without Cu or Zn are shown in Figs. 1 to 3. The general effects of Cu, Zn and Fe were to prolong the lag periods, to increase the generation time and to decrease the growth yields.

Effect of Cu and Zn on the sulfur-oxidizing activity

Various strains were grown on sulfur with and without 10 mM $CuSO_4$ or 50 mM $ZnSO_4$ and cells were harvested after 7 days for the cell yield and sulfur-oxidizing activity determinations with and without 167 mM $CuSO_4$ or 417 mM $ZnSO_4$.

Table 5 shows the results of cells grown with and without Cu. The sulfur-oxidizing activity of control cells was affected by Cu and Zn differently among strains. SM-3, SM-4, SM-6, SM-7 and SM-8 were relatively more resistant to Cu and Zn inhibition of the activity (80-100% of control activity) than Tt, Tf-1, Tf-2, SM-1 and SM-5. SM-1 and SM-5 were particularly sensitive to these metals both on the activity and growth (Tables 2 and 3). SM-3, SM-4, SM-6 and SM-7 were tolerant of these metals both on the activity and growth. SM-8, which was sensitive to the metals on growth, particulary to Cu, was not affected much on its sulfur-oxidizing activity.

Growth in the presence of 10 mM $CuSO_4$ increased the Cu resistance of cells on the sulfur-oxidizing activity in all the isolates tested (SM-8 did not grow). The Zn resistance generally decreased by the growth with Cu except in SM-5 and SM-6. Laboratory strains, Tt and Tf-1, became more sensitive to both Cu and Zn.

It should be mentioned that the effect of $ZnSO_4$ on the sulfur-oxidizing activity not reported in the table is that the lag period is increased extensively before a linear oxygen consumption is observed.

Table 6 shows the results with cells grown on sulfur with and without Zn, but this time at 28°C instead of 25°C because of an incubator problem, i.e., the sulfur-growing cultures inhibiting the growth of iron-growing cultures. Growth yield was generally higher at 28°C.

The sulfur-oxidizing activities of control cells grown at 28°C were considerably more sensitive to Cu and Zn than those cells grown at 25°C with the exception of Tf-2, SM-1 and SM-5 to Cu, as shown in Table 6. All strains became more sensitive to Zn, some extremely sensitive (e.g. SM-4). SM-3, SM-4, SM-6, SM-7 and SM-8, considered as resistant to the metal effect at 25°C, are now indistinguishable from other sensitive strains in their sensitivity to the metals on their sulfur-oxidizing activities.

Growth in the presence of 50 mM $ZnSO_4$ increased the Cu and Zn resistance of cells on the sulfur-oxidizing activity in all isolates, but decreased the resistance in some laboratory strains, Tt (Zn), Tf-1 (Cu) and Tf-2 (Zn).

II. Iron grown-cells

General growth characteristics

An initial growth study of various strains was carried out both in the M9K medium and in the 9K medium (6) at pH 2.3, the latter having higher concentrations of salts than the former. The $FeSO_4 \cdot 7H_2O$ concentration was set at 33.3 g/L (0.119 M) in both media.

As shown in Table 7 Tf-1, Tf-2, SM-3 and SM-4 all grew rapidly after a very short lag (2-3 hours) with a generation time of 5-7.5 hours and oxidized almost all the Fe^{2+} iron to the Fe^{3+} form (0.11 M Fe^{3+}) within 25 hours of shaking at 25°C, both in M9K and 9K media. Sometimes, particularly in the 9K medium growth curves showed biphasic lines, the first rapid growth phase followed by the slower phase leading to the depletion of Fe^{2+}.

SM-1, SM-2, SM-5 and SM-8 were slower due to extended lag periods or longer generation time or both. The 9K medium particularly extended the lag periods of SM-2 and SM-5. Growth time for complete Fe^{2+} oxidation varied from 25 hours in the fast growth group to 60 to over 100 hours in the slow growth group.

Growth yield either in mg protein or mg wet cells fluctuated considerably

among strains (Table 7). Because of difficulty in removing ferric iron precipitate completely from cells the mg protein values are considered more reliable. Both Tf-1 and Tf-2 had high growth yields, but surprisingly SM-5, one of the slow growers, showed the highest growth yield. SM-2 had the lowest growth yield (2 mg protein/L) and the lowest iron-oxidizing activity per mg protein making it the strain with the lowest iron-oxidizing activity recoverable from 1 L of culture. Both growth yields and activities for the same strains fluctuated to some extent among experiments.

Effect of $CuSO_4$ on the growth

$CuSO_4$ at increasing concentrations (1-100 mM) increased both the length of lag period and the generation time as shown in Table 8 and Figs. 4 and 5. Often in the presence of Cu there was an initial minor growth, normally corresponding to one cell division (Figs. 4 and 5), followed by an extended lag period before a major growth phase. In Tf-1 with 100 mM Cu the major growth phase was followed by a slower growth phase leading to the complete oxidation of Fe^{2+} to Fe^{3+} (Fig. 4).

In all strains the growth was little influenced by $CuSO_4$ up to 10 mM. $CuSO_4$ at 30 mM had little effect on Tf-2, SM-1 and SM-5, but increased the lag period considerably of Tf-1, SM-3, SM-4, and SM-8. $CuSO_4$ at 50 mM extended the lag period considerably of Tf-2 in addition to Tf-1, SM-3, SM4 and SM-8, but affected SM-1 and SM-5 much less. $CuSO_4$ at 100 mM extended the lag period of all strains to 150 hours or longer except SM-1 and the generation time to two times (Tf-1, SM-1, SM-5, SM-8), four times (Tf-2), eight times (SM-4) and twelve times (SM-3). SM-2, a slow grower, grew more slowly than in experiments in Table 7, but the growth was little affected by Cu even at 100 mM. Three slow-growers (SM-1, SM-2 and SM-5) are considered more tolerant of Cu than other strains in their growth.

Effect of $ZnSO_4$ on the growth

The effect of $ZnSO_4$ on the growth of various strains is shown in Table 9. SM-1 and SM-2 were not included due to difficulty in their growth. The strains tested were all remarkably tolerant of Zn during growth on ferrous iron. At a concentration of 100 mM Zn had little effect on the growth of SM-4 and SM-8 and increased the lag period of SM-5 two times and both the lag period and generation time of SM-3 three times. Even at 150 mM Zn had little effect on Tf-1 and SM-4, only doubled the lag period of Tf-2 and the lag period and generation time of SM-8, and quadrupled the lag period and generation time of SM-3. Only SM-5 did not grow after 140 hours, although it grew at 100 mM Zn with the same generation time as the control after a lag period only twice that of control.

Effect of Fe^{2+} concentrations on Fe^{2+}-oxidizing activity

The concentration of Fe^{2+} required for the half maximal velocity (1/2 V_{max}) was obtained as K_m by double reciprocal plots of Fe^{2+}-oxidizing activity versus Fe^{2+} concentrations and extending the line to the X-axis (Figs. 6,7). The K_m values for Fe^{2+} were very similar among various strains (Table 10) and were around 0.3-0.5 mM, except with Tf-1 and Tf-2, the two lab strains, and SM-2, the values were close to 1 mM. Sometimes biphasic plots were observed with higher Fe^{2+} concentrations (>1 mM) activating the Fe^{2+} oxidation with higher K_m values (Fig. 7).

Effect of Fe^{3+}, Cu^{2+}, and Zn^{2+} on Fe^{2+}-oxidizing activity

Fe^{3+} inhibited the Fe^{2+}-oxidizing activity of all strains competitively with respect to the Fe^{2+} concentration (Fig. 6 and Table 10) in agreement with Kelly & Jones (7). K_i values for Fe^{3+} were relatively constant among strains, around 2 mM Fe^{3+}, except with SM-3 where the slope replot was biphasic with two K_i values. In all the strains therefore increasing Fe^{3+} concentrations

increased the apparent K_m values for Fe^{2+} (i.e. the concentration required for half the maximal velocity).

The inhibition by Cu^{2+} was complex (Fig. 7 and Table 10). At high Fe^{2+} concentrations, Cu^{2+} inhibited the Fe^{2+} oxidation competitively, changing only the slope of the double reciprocal plots, except with SM-5. In the presence of Cu^{2+} the double reciprocal plots of most strains became distinctly biphasic at low Fe^{2+} concentrations with corresponding intercept changes. With Tf-1 at low Fe^{2+} the lines with Cu^{2+} became parallel to the control line without Cu^{2+}, thus changing only the intercept (uncompetitive inhibition). With SM-5 the plots were still biphasic, but both intercept and slope changed at either high Fe^{2+} or low Fe^{2+} (noncompetitive). K_i values in Table 10 represented the measure of effect on the slope (Cu^{2+} concentration required for doubling of the slope) at high Fe^{2+} (K_i slope) and effect on the intercept (Cu^{2+} concentration required for doubling of the intercept) at low Fe^{2+}. The K_i values for Cu^{2+} in Table 10 varied from 30 mM to 155 mM. At high Fe^{2+} the K_i was fairly constant among isolates (30-70 mM), except SM-2. At low Fe^{2+} SM-3 and SM-4 showed higher K_i values (150 and 155 mM Cu^{2+}) compared to others.

The inhibition by Zn^{2+} was similar to Cu^{2+}. All strains were competitively inhibited by Zn^{2+} at high Fe^{2+}. At low Fe^{2+} the inhibition plots of some strains became biphasic with intercept changes.

Comparison of metal effects on growth and on activity

The Ki values of various strains did not relate easily to their response to Cu and Zn during growth (Tables 8 and 9). Under the growth conditions reported the K_i values for high Fe^{2+} concentrations are considered to be more relevant. During the growth on Fe^{2+} SM-1, SM-2 and SM-5 were found to be most tolerant of Cu but the Fe^{2+}-oxidizing activities of SM-1 and SM-5 were sensitive to inhibition by Cu (low K_i values) while that of SM-2 was not. The K_i values for

Cu^{2+} and Zn^{2+} reported in Table 10 are too large to have a major effect on the binding of Fe2+ under the growth experiments. For example SM-4 has a K_m value for Fe^{2+} of 0.46 mM and a K_i value for Cu^{2+} of 30 mM. Since the K_m value increases by a factor of $[I]/K_i$ in competitive inhibition by an inhibitor I at a concentration [I], the highest Cu concentration used for growth experiment of 100 mM, the apparent K_m value will go up to 2 mM Fe^{2+} (0.46 mM + 0.46 mM x 100 mM/30 mM). In the growth medium containing 33.3 g $FeSO4 \cdot 7H_2O$/L (120 mM) the Fe^{2+} oxidizing system of bacteria will still be 98% saturated with Fe^{2+}, since $[S]/(K_m + [S])$ will be 120 mM/2 mM + 120 mM), where [S] is the concentration of substrate, Fe^{2+}. The system therefore will be oxidizing Fe^{2+} almost without inhibition.

The metal tolerance of T. ferrooxidans during growth must involve not only the inhibition by metals of the Fe^{2+}-oxidizing activity, but also the inhibition of other biochemical reactions essential for growth which are probably more sensitive to metal inhibition if exposed. The extended lag period is very likely the period to detoxify or exclude the metals from the inside of cells so that the necessary reactions for cell divisions can take place. The complex nature of Cu and Zn inhibition particularly at low Fe^{2+} concentrations (Table 10) may have a significant implication on the metal tolerance in bacterial leaching where the concentration of Fe^{2+} will be much lower than our growth conditions (120 mM Fe^{2+}).

Continuing work

Adaptation of sulfur-growing and iron-growing strains to Cu and Zn is continuing. Our preliminary results indicate that the lag periods can be shortened during adaptation. These adapted cells should provide us more precise information on the metal tolerance of various strains.

In conclusion we have shown that fresh isolates from the mine sites are

generally more tolerant of metals when grown on sulfur than laboratory strains which were maintained in laboratory media for a long period of time.

ACKNOWLEDGEMENTS

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Table 1. Growth characteristics, sulfur-oxidizing activity and color of various strains grown at 25°C on sulfur for 7 days.

Strains	Lag period days	Generation time hours	Growth yield/L mg protein	Growth yield/L mg wet cells	Activity nmol O_2/min per mg protein	Color of packed cells
Tt	3	8.0	51	400	220	milky white
Tf-1	2	7.8	45	250	187	bright yellow
Tf-2	2	8.7	41	250	224	bright yellow
SM-1	3	8.9	67	250	208	brownish grey
SM-3	3	9.5	32	100	51	bright yellow
SM-4	3	8.1	37	125	76	bright yellow
SM-5	2	7.8	47	250	89	milky white
SM-6	3	8.4	41	400	273	yellow
SM-7	2	8.9	59	300	196	brownish grey
SM-8	2	8.4	62	400	65	milky pale yellow

SM-2: No growth on sulfur.

Table 2. Effect of $CuSO_4$ on the growth of various strains grown at 25°C on sulfur for 7 days.

Strains	Growth (% of control in mg protein/L)		
	10 mM $CuSO_4$	30 mM $CuSO_4$	50 mM $CuSO_4$
Tt	97	22	15
Tf-1	62	0.5	0.5
Tf-2	81	0.5	0.5
SM-1	51	1.6	1.1
SM-3	56	80	9.3
SM-4	80	87	73
SM-5	41	2.8	1.8
SM-6	34	37	12.5
SM-7	82	51	10
SM-8	10	7.7	1.1

Cu-tolerant: SM-4 (most tolerant), Tt, SM-3, SM-6, SM-7
All produce black CuS, tightly bound to SM-6 cells.

Cu-sensitive strains: Tf-1, Tf-2, SM-1, SM-5, SM-8 (most sensitive).
None produces CuS.

Table 3. Effect of $ZnSO_4$ on the growth of various strains grown at 25°C on sulfur for 7 days.

Strains	Growth (% of control in mg protein/L)		
	50 mM $ZnSO_4$	100 mM $ZnSO_4$	200 mM $ZnSO_4$
Tt	32	28	1.2
Tf-1	48	15	4.6
Tf-2	28	5.0	2.5
SM-1	2.8	3.5	0.8
SM-3	92	15	13
SM-4	22	5.1	2.9
SM-5	2.4	2.8	1.0
SM-6	62	29	34
SM-7	99	69	52
SM-8	23	4.3	0.9

Zn-tolerant strains: SM7 and SM-6 (most tolerant), SM-3, Tf-1, SM-4, Tf-2, Tt, SM-8 (least tolerant)

Zn-sensitive strains: SM-1, SM-5

Table 4. Effect of $FeSO_4$ on the growth of various strains grown at 25°C on sulfur for 7 days.

Strains	Growth (% of control in mg protein/L)		
	50 mM $FeSO_4$	100 mM $FeSO_4$	200 mM $FeSO_4$
Tt	74	67	27
Tf-1	96	93	55
Tf-2	62	46	25
SM-1	83	52	26
SM-3	70	48	37
SM-4	74	44	33
SM-5	102	95	40
SM-6	72	35	25
SM-7	65	31	14
SM-8	119	68	21

All strains are Fe^{2+} tolerant.

Table 5. Effect of Cu and Zn on the sulfur-oxidizing activity of various strains grown at 25°C on sulfur for 7 days with and without 10 mM $CuSO_4$.

	Activity (% of control without Cu or Zn)			
	167 mM Cu		417 mM Zn	
Strains	Cells (0 mM Cu)	Cells (10 mM Cu)	Cells (0 mM Cu)	Cells (10 mM Cu)
Tt	74	70	57	54
Tf-1	79	65	48	42
Tf-2	70	73	40	37
SM-1	53	77	43	32
SM-3	95	100	88	73
SM-4	88	95	90	67
SM-5	69	76	36	48
SM-6	97	110	77	95
SM-7	92	96	81	72
SM-8	84		92	

All isolates tested become more Cu-resistant after growth with 10 mM Cu.

Table 6. Effect of Cu and Zn on the sulfur-oxidizing activity of various strains grown at 28°C on sulfur for 7 days with and without 50 mM $ZnSO_4$.

Strains	Activity (% of control without Cu or Zn)			
	167 mM Cu		417 mM Zn	
	Cells (0 mM Zn)	Cells (50 mM Zn)	Cells (0 mM Zn)	Cells (50 mM Zn)
Tt	67	73	51	42
Tf-1	73	64	12	22
Tf-2	82	88	35	29
SM-1	56	85	15	19
SM-3	62	77	23	24
SM-4	72	78	9	21
SM-5	69	86	12	23
SM-6	58	86	36	43
SM-7	63	76	31	33
SM-8	60	74	13	30

All isolates become more Cu- and Zn-resistant after growth with 50 mM Zn.

Table 7. Growth characteristics and iron-oxidizing activity of various strains grown at 25°C on ferrous iron.

Strains	Growth medium	Lag period hours	Generation time hours	Growth time** hours	Growth yield/L mg protein	Growth yield/L mg wet cells	Activity (V_{max}) per mg protein nmol O_2/min
Tf-1	M9K	2-3	6.8	25	8.8	83	1250
	9K	2-3	5.5(10)*	25			
Tf-2	M9K	2-3	6.8	25	15.4	80	1250
	9K	2-3	5.0(10)*	25			
SM-1	M9K	15	9.6(35)*	60	2.6	20	680
	9K	2-3	12.0(29)*	55			
SM-2	M9K	2-3	18.8	70	2.0	25	190
	9K	80	18.0	130			
SM-3	M9K	2-3	6.0(8.5)*	25	7.2	49	230
	9K	2-3	6.0(10)*	25			
SM-4	M9K	2-3	7.5	25	3.4	40	1740
	9K	2-3	7.0	25			
SM-5	M9K	20	21.0	130	49.1	332	800
	9K	225	24.4	275			
SM-8	M9K	10	45.6	140	5.0	28	690
	9K	10	30.4	100			

*Generation time during the second and slower growth phase before Fe^{2+} depletion.
**Incubation time at 25°C for complete oxidation of Fe^{2+} to Fe^{3+}.

Fast growers: Tf-1, Tf-2, SM-3, SM-4
Slow growers: SM-1, SM-2, SM-5, SM-8

Table 8. Effect of $CuSO_4$ on the growth of various strains on ferrous iron at 25°C.

Strains	Lag period (LP) or generation time (Gt) in hours					
	$CuSO_4$ Concentrations					
	0 mM	1 mM	10 mM	30 mM	50 mM	100 mM
Tf-1 (Lp)	4	4	8	(12)* 94	(12)*156	(12)*244
(Gt)	7	7	10	(30)* 10	(30)* 11	(30)*16(110)**
Tf-2 (Lp)	2	2	2	3	(4)* 90	(4)*155
(Gt)	8	8	8	9	(19)* 11	(19)* 29
SM-1 (Lp)	12	12	12	17	30	88
(Gt)	13	13	13	13	22	32
SM-2 (Lp)	(2)*208		(2)*208		(2)*208	(2)*208
(Gt)	(60)* 45		(64)* 46		(64)* 45	(64)* 60
SM-3 (Lp)	2	2	2	(2)*134	(2)*220	(2)*300
(Gt)	6	6	10(15)**	(24)* 14	(24)* 28	(24)* 70
SM-4 (Lp)	4	4	4	(4)* 80	(4)*140	(4)*280
(Gt)	7	7	11	(15)* 22	(15)* 28	(15)* 57
SM-5 (Lp)	25	25	25	28	38	(50)*160
(Gt)	17(40)**	17(40)**	17(40)**	20(80)**	28(80)**	(107)* 33
SM-8 (Lp)	6	6	6	36	64	158
(Gt)	17	17	17	23	23	36

*Lag period and generaton time for the initial minor growth before an extended lag followed by the major growth.
**Generation time during the slower growth phase before Fe^{2+} depletion.

Table 9. Effect of $ZnSO_4$ on the growth of various strains on ferrous iron at 25°C.

Strains	Lag period (Lp) or generation time (Gt) in hours				
	$ZnSO_4$ concentrations				
	0 mM	10 mM	50 mM	100 mM	150 mM
Tf-1 (Lp)	2				2
(Gt)	8				9
Tf-2 (Lp)	18				32
(Gt)	9				9
SM-3 (Lp)	2	3	3	6	8
(Gt)	10	10	21	34	46
SM-4 (Lp)	2	2	3	3	4
(Gt)	12	12	12	12	15
SM-5 (Lp)	38	68	95	80	<140
(Gt)	11	16	15	11	
SM-8 (Lp)	38		50	57	63
(Gt)	18		18	20	29

Table 10. Effect of Fe^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} concentrations on the ferrous iron oxidizing activity of various strains.

Strain	K_m	Inhibition							
		Fe^{3+}		Cu^{2+}			Zn^{2+}		
	mM Fe2+	Type*	K_i (mM)	Type* High Fe^{2+}	Type* Low Fe^{2+}	K_I (mM)	Type* High Fe^{2+}	Type* Low Fe^{2+}	K_i (mM)
Tf-1	1.2	C	1.5	C		83(s)**	C		75(s)
					UC	50(i)**		UC	80(i)
Tf-2	0.95	C	1.8	C		130(s)	C		142(s)
					NC	90(i)			
SM-1	0.35	C	1.9	C		54(s)	C		77(s)
SM-2	1.0	C	2.0	C		130(s)	C		300(s)
SM-3	0.20	C	1.2	C		70(s)	C		180(s)
	0.59		10.0		NC	150(i)		NC	180(i)
SM-4	0.46	C	2.0	C		30(s)	C		46(s)
					NC	155(i)		NC	215(i)
SM-5	0.43	C	1.5	NC		50(s)	C		55(s)
					NC	75(i)		NC	160(i)
SM-8	0.33	C	2.0	C		68(s)	C		116(s)
					NC	60(i)		NC	120(i)

*Type C: Competitive; no intercept change, only slope change.
UC: Uncompetitive; no slope change, only intercept change.
NC: Noncompetitive; both intercept and slope change.
High Fe^{2+}, low Fe^{2+}: Biphasic pattern with one type of inhibition at high Fe^{2+} concentrations and another type at low Fe^{2+} concentrations.

**s = slope, i = intercept.

Figure Legends

Fig. 1. Growth of T. thiooxidans ATCC 8085 (Tt) on sulfur with and without Cu^{2+}.

Fig. 2. Growth of SM-7 on sulfur with and without CU^{2+}.

Fig. 3. Growth of Tt and SM-7 on sulfur with and without Zn^{2+}.

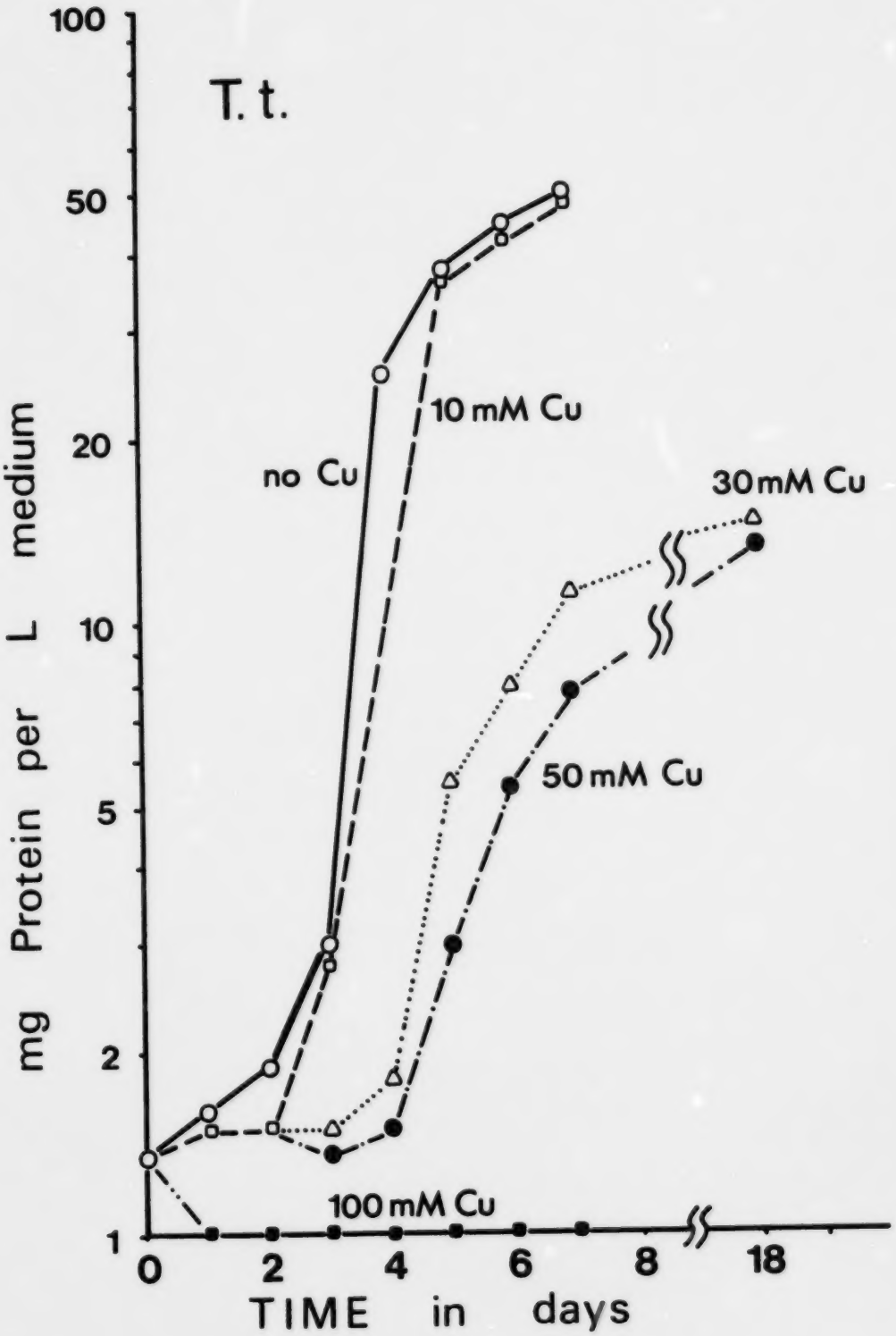
Fig. 4. Growth of T. ferrooxidans ATCC 13661 (Tf-1) on Fe^{2+} with and without Cu^{2+}.

Fig. 5. Growth of SM-4 on Fe^{2+} with and without Cu^{2+}.

Fig. 6. Effect of Fe^{2+} concentration on the Fe^{2+} oxidizing activity of SM-4 and its inhibition by Fe^{3+}.

Fig. 7. Effect of Fe^{2+} concentration on the Fe^{2+} oxidizing activity of SM-3 and its inhibition by Cu^{2+}.

Figure 1


T. t.
100
50
20
10
5
2
1
mg Protein per L medium
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10 mM Cu
30 mM Cu
50 mM Cu
100 mM Cu
0
2
4
6
8
18
TIME in days

Figure 2

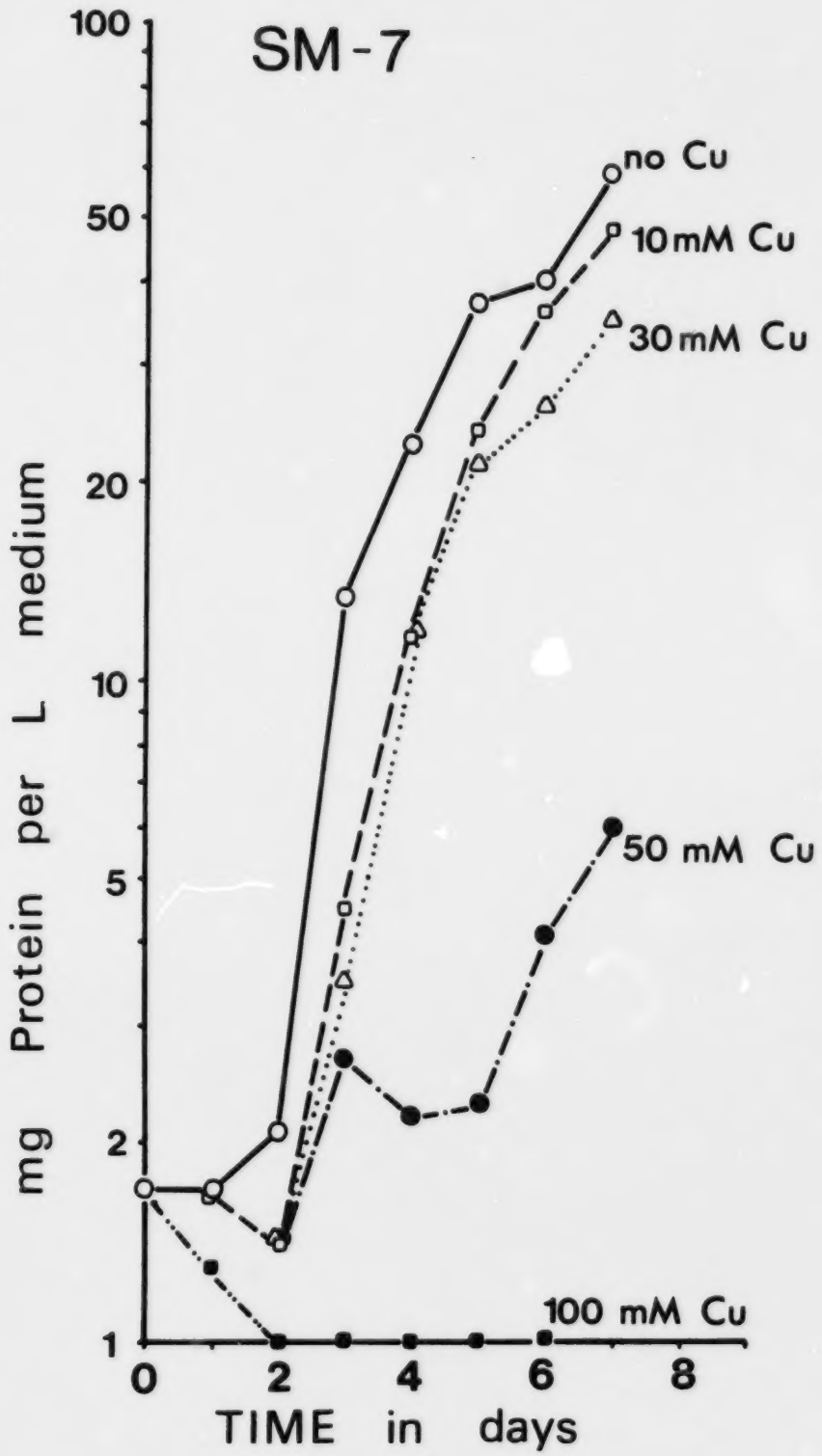

SM-7
100
50
20
10
5
2
1
mg Protein per L medium
no Cu
10 mM Cu
30 mM Cu
50 mM Cu
100 mM Cu
0
2
4
6
8
TIME in days

Figure 3

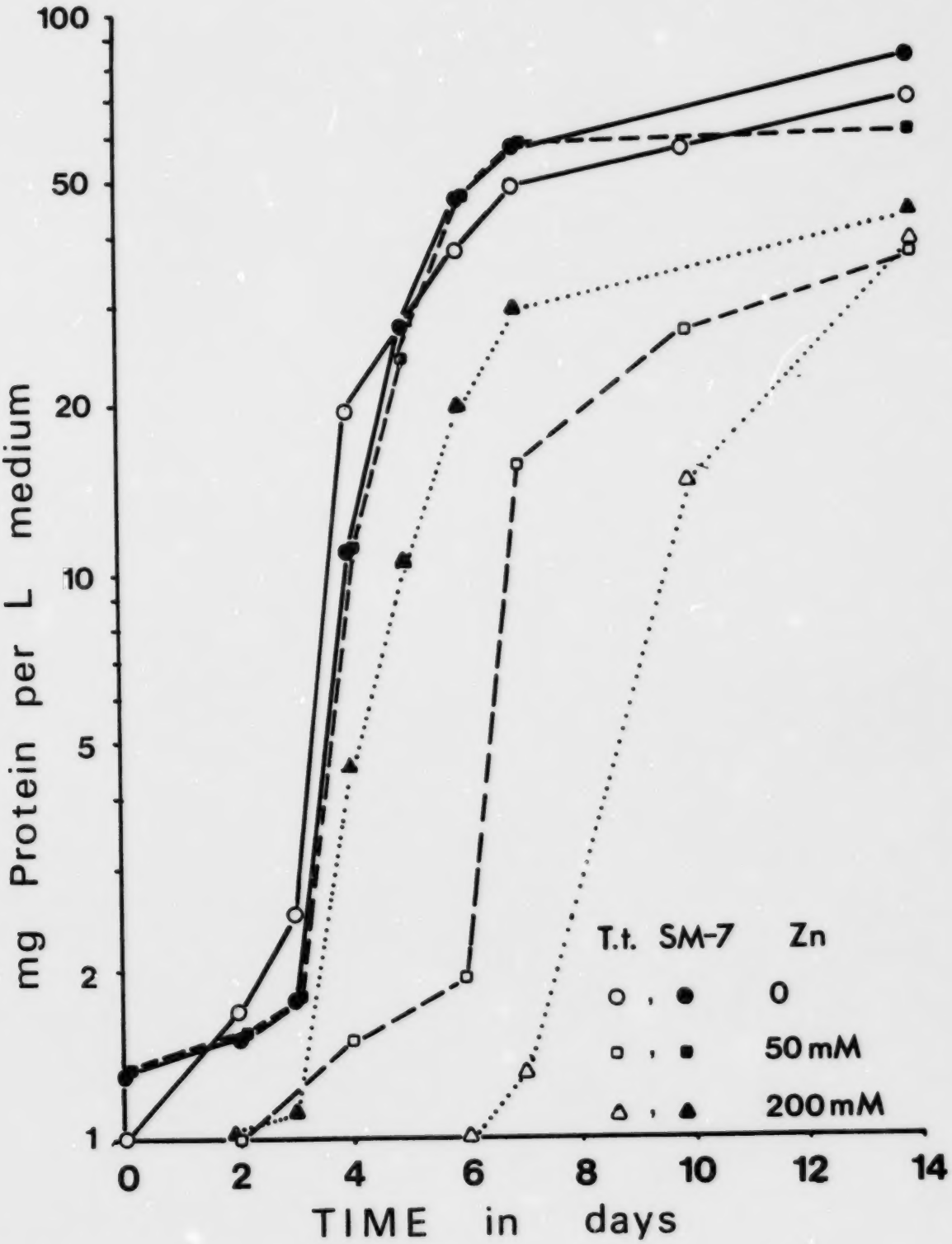

100
50
20
10
5
2
1
mg Protein per L medium
0
2
4
6
8
10
12
14
TIME in days
T.t. SM-7 Zn
0
50 mM
200 mM

Figure 4

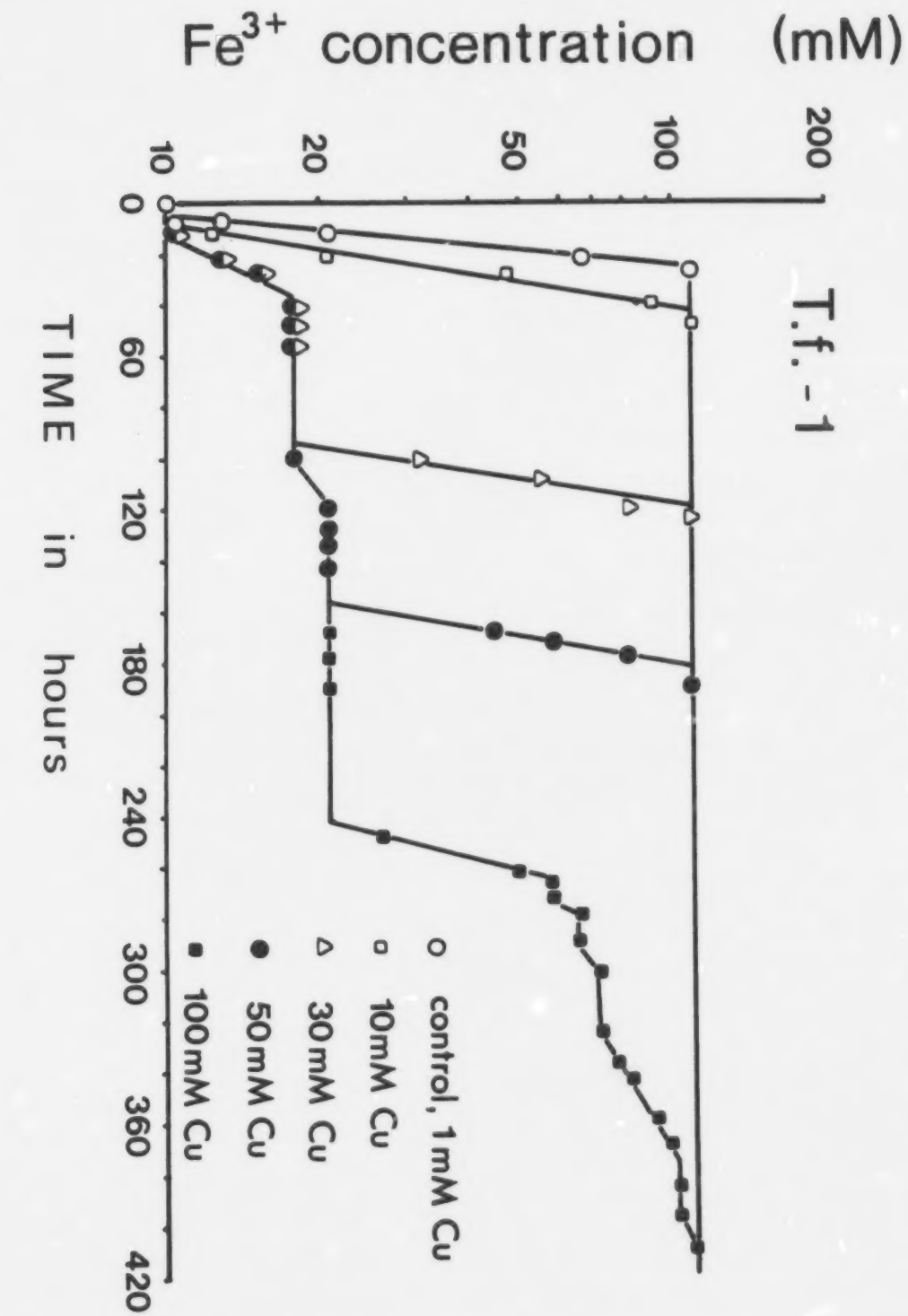

Fe3+ concentration (mM)
200
100
50
20
10
0
T.f. - 1
TIME in hours
0
60
120
180
240
300
360
420
control, 1 mM Cu
10mM Cu
30 mM Cu
50 mM Cu
100 mM Cu

Figure 5

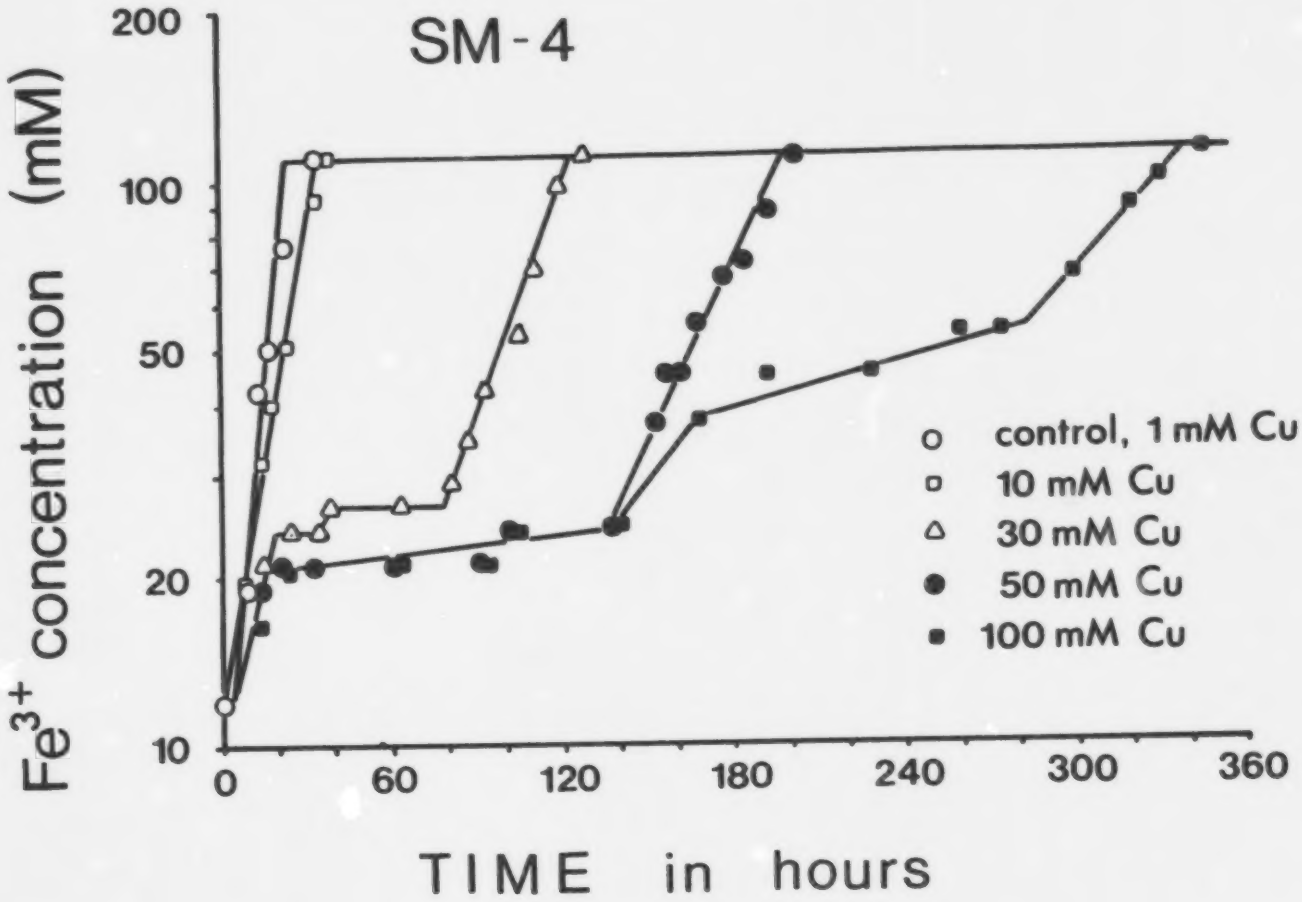

SM-4
200
100
50
20
10
Fe^{3+} concentration (mM)
0
60
120
180
240
300
360
TIME in hours
control, 1 mM Cu
10 mM Cu
30 mM Cu
50 mM Cu
100 mM Cu

Figure 6

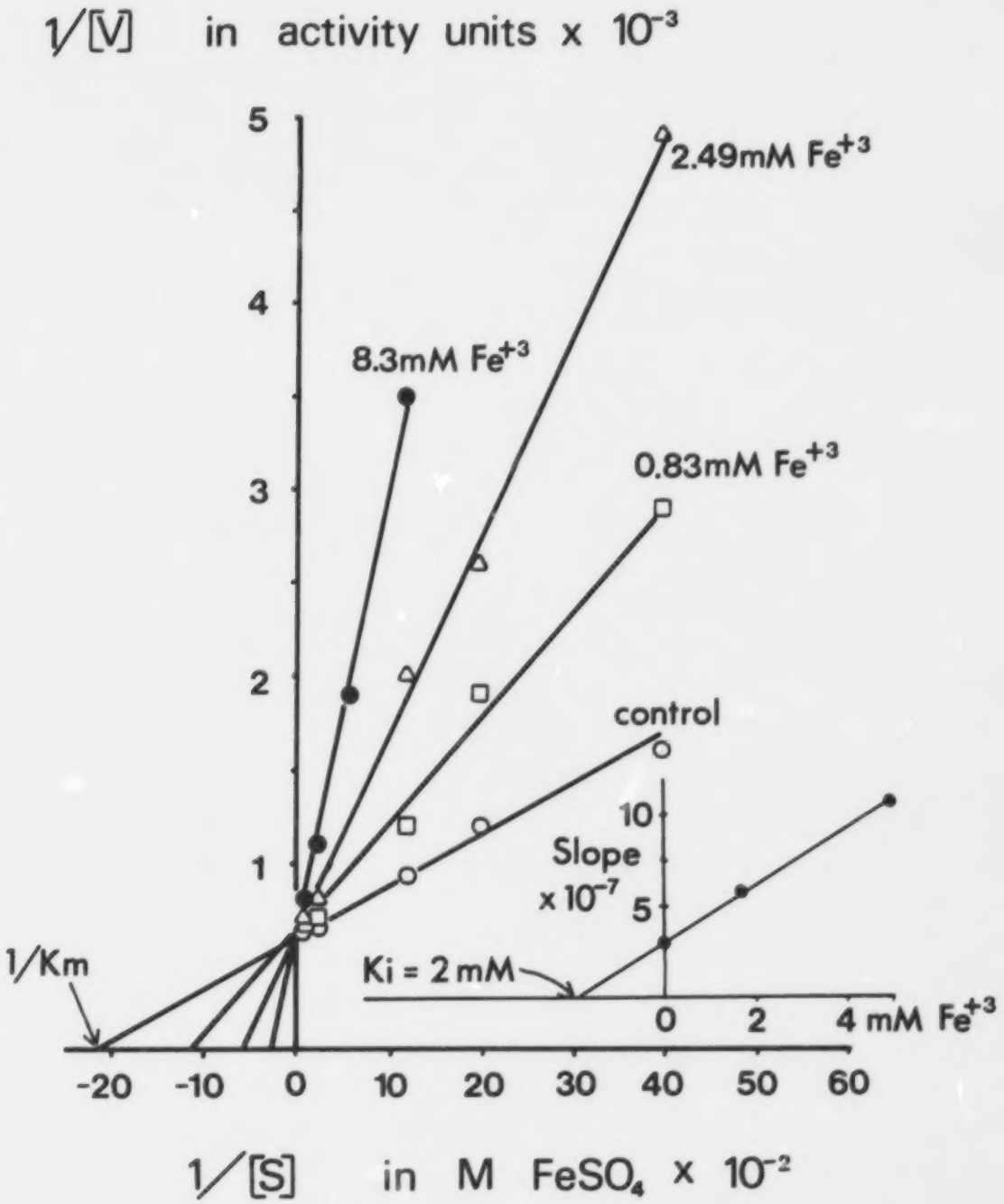
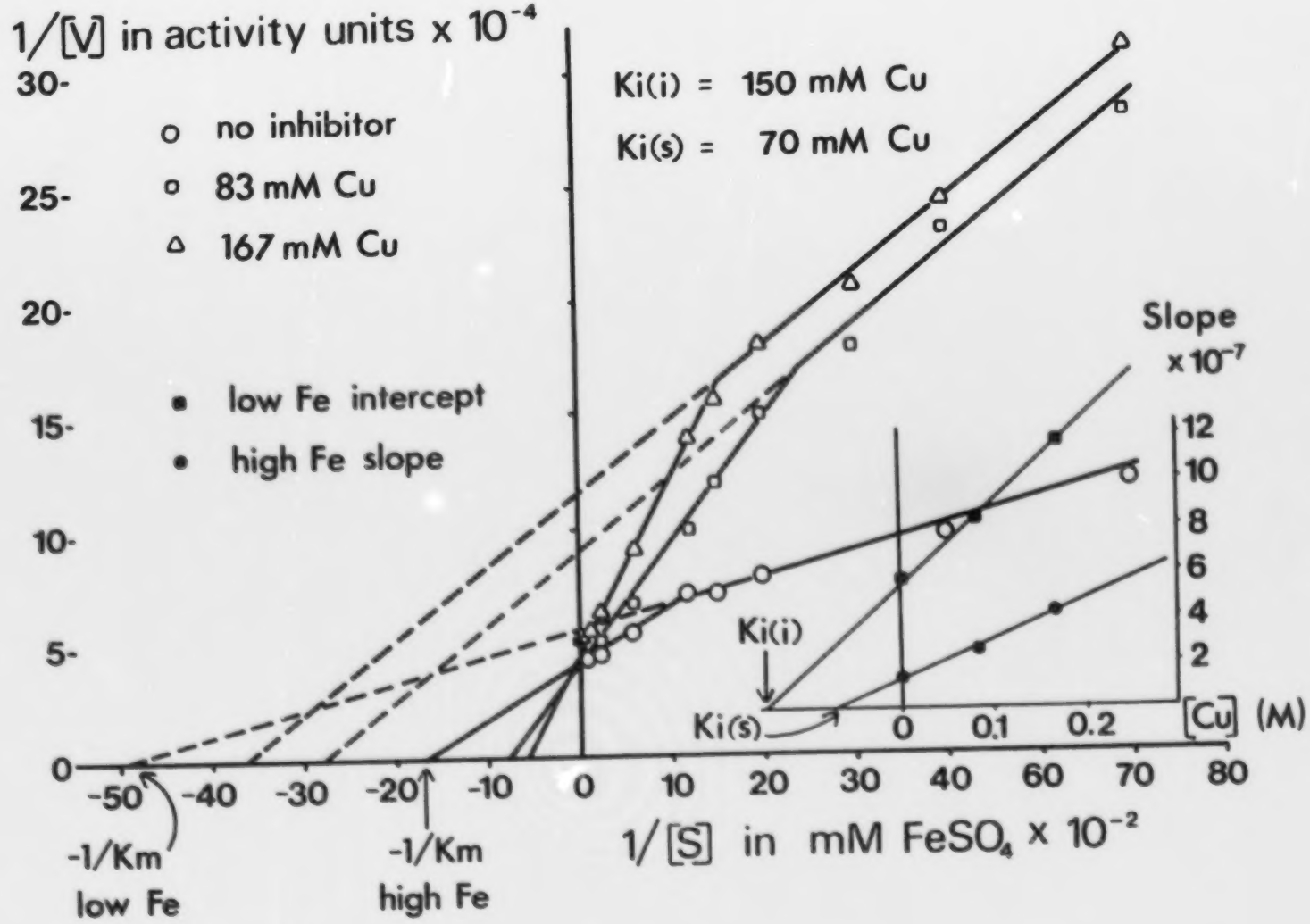

1/[V] in activity units x 10^{-3}
5
4
3
2
1
2.49mM Fe^{+3}
8.3mM Fe^{+3}
0.83mM Fe^{+3}
control
10
Slope
x 10^{-7}
5
1/Km
Ki = 2 mM
0
2
4 mM Fe^{+3}
-20
-10
0
10
20
30
40
50
60
1/[S] in M $FeSO_4$ x 10^{-2}

Figure 7


1/[V] in activity units x 10^{-4}
30-
25-
20-
15-
10-
5-
0
o no inhibitor
o 83 mM Cu
Δ 167 mM Cu
• low Fe intercept
• high Fe slope
Ki(i) = 150 mM Cu
Ki(s) = 70 mM Cu
Slope x 10^{-7}
12
10
8
6
4
2
Ki(i)
Ki(s)
0
0.1
0.2
[Cu] (M)
-50
-40
-30
-20
-10
0
10
20
30
40
60
70
80
-1/Km low Fe
-1/Km high Fe
1/[S] in mM $FeSO_4$ x 10^{-2}

STAGE II

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EXECUTIVE SUMMARY

We have grown various laboratory strains (Tf - Thiobacillus thiooxidans ATCC 8085, Tf-1 - Thiobacillus ferrooxidans ATCC 13661 and Tf-2 - T. ferrooxidans ATCC 19589) and mine isolates (SM-1 to SM-8, only SM-6 and SM-7 being T. thiooxidans and the rest T. ferrooxidans) on elemental sulfur (except SM-2) and ferrous iron (only T. ferrooxidans) in the presence of various metals to study the level of metal tolerance and adaptation to toxic concentration of metals. Both control cells and metal-adapted cells were studied for their oxidation activity and metal inhibition.

Tt, SM-4, SM-6 and SM-7 adapted to growth on sulfur in the presence of 50 and 100 mM $CuSO_4$ became Cu-dependent for good growth, produced black or grey cells whose sulfur-oxidizing activity was either tolerant of Cu or activated by a normally inhibitory concentration of Cu (167 mM). All strains except SM-2 adapted to growth on Fe^{2+} in 50 mM $CuSO_4$. The K_m values for Fe^{2+} were high with laboratory strains (Tf-1 and Tf-2) and increased upon adaptation, while those with mine isolates (except SM-2) were low and remained low or became lower upon adaptation. The Fe^{2+}-oxidizing activity of Cu-adapted cells was activated by normally inhibitory concentrations of metals only in mine isolates (Cu and Zn in SM-1, SM-3, SM-4, SM-5; Fe^{3+} in SM-4 and SM-5).

All strains except SM-1 adapted to growth on Fe^{2+} with 150 mM $ZnSO_4$. High K_m values for Fe^{2+} of Tf-1, Tf-2 and SM-2 remained high after Zn adaptation, while those low values of other mine isolates remained low or became even lower upon Zn adaptation. The Fe^{2+} oxidation by Zn-adapted mine isolates was also activated by metals (Zn and Cu in SM-3, SM-4, SM-8; Fe^{3+} in SM-3 and SM-4) or was much less inhibited by Zn and Cu (SM-5).

Growth of all strains was sensitive to $AgNO_3$ and $HgCl_2$. In the sulfur medium Tt, SM-4 and SM-6 grew at 10 μM Ag, Tf-2, SM-3, SM-7 and SM-8 at 5 μM Ag

and Tf-1, SM-1 and SM-5 only at 1 μM Ag. The sulfur oxidation by Tt, SM-1, SM-6, SM-7 and SM-8 was inhibited by 50% at around 100 μM Ag and that by Tf-1, Tf-2, SM-3, SM-4 and SM-5 at around 50 μM Ag. In the Fe^{2+} medium Tf-1, SM-3 and SM-4 grew in 1 μM Ag; SM-2 and SM-5 grew in 1 μM Ag only sometimes; Tf-2 and SM-8 grew in 0.1 μM Ag; and SM-1 did not grow in 0.1 μM Ag. Ag was an uncompetitive inhibitor of Fe^{2+} oxidation with K_i values for control cells of 125-525 μM Ag decreasing to 30-260 μM Ag for Ag-adapted cells. A time-dependent increase in inhibition by Ag was observed with both sulfur and Fe^{2+} oxidations.

Tt, Tf-1, Tf-2, SM-6, SM-7 and SM-8 grew at 5 μM Hg in the suflur medium; SM-1, SM-3, SM-4, and SM-5 grew at 1 μM Hg, of which only SM-3 and SM-5 adapted to 5 μM Hg. The sulfur oxidation was inhibited by 50% at 2-5 μM Hg. In the Fe^{2+} medium only SM-4 and SM-8 grew in 1 μM Hg and the others grew at 0.1 μM Hg. The inhibition of Fe^{2+} oxidation by Hg was uncompetitive with K_i values of 50-210 μM with control cells, converging to 50-80 μM Hg with Hg-adapted cells. The inhibition was time-dependent.

SM-4, SM-6, SM-7 and SM-8 grew well at 100 μM Na_2MoO_4 in the sulfur medium, while the others except SM-3 adapted to 100 μM Mo. SM-3 grew only at 50 μM Mo. Growth yields were normally higher in the presence of Mo. Cells grown at 100 μM Mo were uniquely blue and showed higher activity and higher Mo tolerance in the sulfur oxidation. Mo at 1 mM inhibited the sulfur oxidation by 8-53% in control cells and even less at 5 mM. In the Fe^{2+} medium Tf-2, SM-2, SM-3, SM-4, SM-5 grew at 1 mM Mo and Tf-1, SM-1 and SM-8 grew at 0.1 mM Mo. SM-2, however, stopped growing after 2 transfers. There was no stimulation of growth or increased activity by Mo in the Fe^{2+} medium. SM-3 was by far the most tolerant of Mo in Fe^{2+} oxidation with K_i values higher than 10 mM Mo. Inhibition by Mo of Fe^{2+} oxidation was generally uncompetitive, but often

complex depending on Fe^{2+} and Mo concentrations with K_i values ranging from 2 μM to 1.3 mM (except SM-3).

All sulfur-growing strains except SM-5 grew at 50 μM $NaAsO_2$. SM-5 grew at 10 μM As. Tt, Tf-2, SM-1, SM-3, SM-4, SM-6 and SM-7 adapted to 50 μM As, while Tf-1, SM-5 and SM-8 adapted only to 5-10 μM As. Arsenite at 5-10 mM inhibited the sulfur-oxidizing activity of control cells by 50%. Adapted cells had less activity and was more sensitive to As inhibition (50% inhibition by 1 mM As). In the Fe^{2+} medium all strains grew at 1 mM $NaAsO_2$. Inhibition of Fe^{2+} oxidation by As was complex, generally uncompetitive at low As, but at high As becoming competitive (SM-3, SM-4, SM-5) or no or little inhibition (Tf-1, Tf-2, SM-1, SM-2, SM-8). Laboratory strains were more sensitive (K_i 2-17 mM) than mine isoaltes (K_i 20-40 mM) based on low As experiments. Time-dependent inhibition was observed sometimes.

It is concluded that SM-4 is the most tolerant of all the metals tested among T. ferrooxidans both in the Fe^{2+} medium and sulfur medium, having only a low tolerance to Hg (1 μM) in the sulfur medium. SM-3 is the next tolerant having low tolerance to Hg and Mo in the sulfur medium and to Hg (0.1 μM) in the Fe^{2+} medium. SM-5 was sensitive to all metals in the sulfur medium and to Ag and Hg in the Fe^{2+} medium. SM-2 was sensitive to Cu and SM-1 to Zn and both were sensitive to Ag and Hg in the Fe^{2+} medium. SM-2 did not grow on sulfur and SM-1 was sensitive to most metals in the sulfur medium. Laboratory strains, Tf-1 and Tf-2, were not as tolerant as many mine strains to most metals. All the T. thiooxidans strains were tolerant of all the metals tested, but SM-6 and SM-7 generally showed shorter generation times after adaptation to inhibitory concentrations of metals than Tt and Tt became sensitive to Hg inhibition of activity after Hg adaptation.

MATERIALS AND METHODS

Bacterial strains

Thiobacillus thiooxidans ATCC 8085 (Tt), Thiobacillus ferrooxidans ATCC 13661 (Tf-1) and T. ferrooxidans ATCC 19859 (Tf-2) were the laboratory strains used as controls. SM-1, SM-2, SM-3, SM-4, SM-5, SM-6, SM-7 and SM-8 were isolated from water samples from various locations in the South Main Shaft of the Flin Flon mine of the Hudson Bay Mining and Smelting Co. Ltd., Manitoba (1). Tt, SM-6 and SM-7 grew only on sulfur as the energy source (T. thiooxidans) and Tf-1, Tf-2, SM-1, SM-3, SM-4 and SM-5 grew both on sulfur and Fe^{2+}, SM-2 grew only on Fe^{2+} (2). All stock cultures were kept at 4°C for long term preservation and at 25°C for the duration of experiments.

Media and growth of bacteria on sulfur

The medium used for the growth on sulfur was the Starkey's No. 1 medium (3): 0.3 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2$, and 18 mg $FeSO_4 \cdot 7H_2O$ per L in distilled water; adjusted to pH 2.3 with H_2SO_4. For $AgNO_3$ experiments $CaCl_2$ was replaced with $CaSO_4$ to avoid AgCl precipitation. Normally a culture was grown in one liter volume in a 2.8 L Fernbach flask at 25°C without agitation after inoculation (2.5% v/v) and covering of surface with powdered precipitated sulfur (BDH Chemicals) (50 g/L) as described (4). For some experiments it was grown in 200 mL volume in a 500 mL Erlenmeyer flask.

Cells were collected by centrifugation after removal of sulfur by filtration through a Whatman No. 1 filter under suction. The cells were washed once in the Starkey's medium and suspended in the same medium (50 mg/mL) for the determination of the sulfur-oxidizing activity within two days.

For the study of metal effect various concentrations of $CuSO_4$, $ZnSO_4$, $AgNO_3$, $HgCl_2$, Na_2MoO_4 and $NaAsO_2$ were mixed with the medium after pH adjustment

to 2.3 if necessary and separate sterilization.

Growth rate on sulfur

Ten mL samples were removed from cultures periodically and bacterial cells were separated from elemental sulfur by filtration through a Whatman No. 1 filter and were collected by centrifugation. The cells were washed three times with the Starkey's No. 1 medium (pH 2.3) and then were boiled for 15 min in 0.1 N NaOH for the determination of total protein concentrations by the method of Lowry et al. (5) using crystalline bovine serum albumin as a standard. Growth curves were plotted on a semi-log scale to obtain the generation time (Gt) and lag period (Lp).

Determination of sulfur-oxidizing activity

The rate of sulfur oxidation was determined by measuring the rate of oxygen disappearance in an Oxygraph (Gilson) with a Clark electrode at 25°C. The standard reaction mixture contained 50 µL of a bacterial cell suspension (50 mg wet cells/mL), 0.3 mL sulfur (320 mg precipitated sulfur, low Fe, BDH per mL 500 ppm Tween 80) and STarkey's medium No. 1 (pH 2.3) in a total volume of 1.2 mL. The sulfur oxidizing activity was expressed as nmol O_2/min. The effect of metals on the activity was studied by including in the reaction mixture various small volumes of concentrated metal solutions adjusted to pH 2.3, if necessary. Again chloride ion was avoided when $AgNO_3$ was used as inhibitor.

Media and growth of bacteria on iron

The medium used for the growth on ferrous iron was a modified 9K (M9K) medium of McCready et al. (6): 0.4g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, and 33.3 g $FeSO_4 \cdot 7H_2O$ per liter and adjusted to pH 2.3 with H_2SO_4. The ferrous sulfate solution (33.3 g/100 mL) was filter sterilized separately. Normally a culture was grown in 100 mL volume (10% inoculum) in a 250 mL Erlenmeyer flask at 25°C on a rotary shaker at 150 rpm. To collect cells for activity studies

larger flasks were used (e.g. 200 mL culture in a 500 mL flask, 130 rpm). Cells were collected by centrifugation (12,000 x g for 10 min) after removal of ferric precipitates by decantation and low speed centrifugation (100 x g for 10 min). The cells were washed twice in water (pH 3 with H_2SO_4) and suspended in the pH 3 water at a concentration of 50 mg wet cells/mL for the determination of Fe^{2+}-oxidizing activity.

For the study of metal effect $CuSO_4$, $ZnSO_4$, $AgNO_3$, $HgCl_2$, Na_2MoO_4 and $NaAsO_2$ solutions were added to the medium after pH adjustment to 2.3 if necessary and separate sterilization. For the study of Ag effect the ferrous sulfate (3.3 g/10 mL) as substrate was treated with $AgNO_3$ (0.1 mL, 0.1 mM) to remove the 0.001% chloride contaminant as AgCl. Three different batches of $FeSO_4 \cdot 7H_2O$ (crystal, certified A.C.S., Fisher) were used during the course of study and affected the growth of various strains and ease of harvesting cells free of ferric precipitates. Light blue dry crystals gave the best results.

Growth rate on iron

Stock cultures were subcultured three times (each subculture at 75-80% consumption of ferrous iron) before the growth curve study of actively growing culture was carried out, with or without metals.

Cell growth was followed by the determination of ferric iron concentration by measuring the yellow color (7) in a Klett-Summerson Photoelectric Colorimeter with a blue (No. 42) filter after 0.5 mL culture sample was mixed with 4.5 mL 1N HCl. Cell growth was also followed by the protein determination (5,8) at the initial stage of study, but was found to be more difficult due to the presence of ferric iron. Therefore the protein determination was used routinely only on the washed cell suspension. Growth curves were plotted on a semi-log scale to obtain the generation time, lag period and total growth time.

Determination of ferrous iron-oxidizing activity

The rate of ferrous iron oxidation by various strains was determined by measuring the rate of oxygen consumption in a Gilson Oxygraph at 25°C. The standard reaction mixture in a total volume of 1.2 mL contained 10 or 25 µL of a bacterial suspension (50 mg wet cells/mL), microliter volumes (3, 6, 10, 50, 150 µL) of $FeSO_4$ (0.1 M, pH 2.3) and M9K medium (no $FeSO_4$, pH 2.3). The metal effect was studied by incorporating in the reaction mixtures various small volumes of concentrated metal solutions. Normally the reaction was initiated with the addition of cells, but for the effects of Ag, Hg, As and Mo the reaction was started with $FeSO_4$ after 5 min preincubation of cells with metals.

Routine assays were carried out with 10 or 50 µL of 0.1 M $FeSO_4$, but for the characterization of each strain the activities at various Fe^{2+} concentrations were plotted in a double reciprocal manner to obtain the maximal velocity, V_{max}, the velocity at the saturating Fe^{2+} concentration, and the K_m, the concentration of Fe^{2+} to produce half the maximal velocity.

Determination of inhibition patterns

Fe^{2+} oxidation rates with and without inhibitors were plotted against the Fe^{2+} concentrations by the double reciprocal method of Lineweaver and Burke (9) and analyzed according to Cleland (10) for the type of inhibition.

Relevant equations are:

No inhibition $\quad 1/v = (K_m/V_{max})(1/S) + 1/V_{max}$

Competititve $\quad 1/v = (K_m/V_{max})(1 + I/K_i)(1/S) + 1/V_{max}$

Uncompetitive $\quad 1/v = (K_m/V_{max})(1/S) + (1/V_{max})(1 + I/K_i)$

Noncompetitive $\quad 1/v = (K_m/V_{max})(1 + I/K_{i\text{-slope}})(1/S) + 1/V_{max})(1 + I/K_{i\text{-intercept}})$

where S = substrate, Fe^{2+}, concentration; I = inhibitor concentration; v = reaction rate or velocity; K_m = Michaelis constant; V_{max} = maximal velocity; K_i

- inhibitor constant (concentration of inhibitor to double the slope or intercept). In the double reciprocal plots of 1/v versus 1/S competitive inhibition increases only the slope, uncompetitive inhibition only the intercept, and noncompetitive inhibition increases both the slope and intercept.

RESULTS

I. Sulfur-grown cells

Adaptation to Cu

Four Cu-tolerant strains; Tt, SM-4, SM-6 and SM-7; were selected for Cu adaptation studies. As shown in Tables 1 and 2 both 50 and 100 mM Cu inhibited the growth of control cultures, but all the 4 strains adapted well to these Cu concentrations.

Table 3 shows the growth yield and color of cells when control and adapted cultures were grown with or without Cu. Cu-adapted cultures all became dependent on the presence of Cu for good growth yield and also acquired a grey to black color of packed cells particularly when grown in the presence of Cu.

The sulfur-oxidizing activity (Table 4) of these cells also indicates that Cu-adapted culture requires the presence of Cu in the medium to produce a high sulfur-oxidizing activity. The sulfur-oxidizing activity of Cu-adapted cells became more tolerant of inhibition by Cu, particularly when grown in the presence of Cu. In some cells the activity was increased, instead of being inhibited, up to over 300%.

Interestingly all the Cu-adapted strains (100 mM $CuSO_4$) grew in the presence of 200 mM $ZnSO_4$ to the same extent as 100 mM $CuSO_4$ and much better than unadapted controls.

Effect of Ag and adaptation to Ag

Three laboratory strains and 7 mine isolates were grown in increasing concentrations of Ag with and without adaptation (Tables 5 and 6). Ag was quite toxic to all the strains, drastically (over 90%) inhibiting the growth in 7 days of Tf-2 at 1 µM and others at 5 µM. With a longer incubation period Tt, SM-4 and SM-6 grew at 10 µM Ag; Tf-2, SM-3, SM-7 and SM-8 at 5 µM Ag; and Tf-1,

SM-1 and SM-5 only at 1 μM Ag. Adaptation to Ag was often difficult and cultures became sometimes more sensitive to Ag inhibition after repeated transfer in the medium containing Ag. Thus the lag period and generation time were not always shortened after adaptation. Only Tf-2 and SM-6 showed shorter generation times after adaptation.

The sulfur-oxidizing activity (Table 7) was also inhibited by Ag, but at higher concentrations than growth, requiring 50-100 μM Ag for 50% inhibition. Ag-adapted cells took longer times for growth, but the growth yield and activity were comparable to controls. Among control cells SM-8, SM-1, Tt, and SM-6 were the most tolerant to Ag inhibition and Tf-1 and Tf-2 were the least tolerant, although the inhibition was never over 80% even at 250 or 500 μM Ag. Only SM-7 (and Tf-1 and Tf-2 to some extent) showed an increased tolerance to Ag^+ inhibition of activity after adaptation and most became more sensitive. Thus the adapted SM-7 was as tolerant of Ag as the most tolerant control strain, SM-8.

The Ag inhibition of sulfur-oxidizing activity was time-dependent after the initiation of reaction with sulfur addition, the reaction slowing down considerably after 10 minutes. Thus in the long incubation (e.g. growth experiments) the sulfur oxidation will be much more strongly inhibited by Ag than indicated in the table. It is nevertheless possible that other reactions essential for growth are more sensitive to Ag inhibition.

Effect of Hg and adaptation to Hg

Hg was found to be even more toxic than Ag to the growth of various strains on sulfur as shown in Tables 8 and 9. None of the strains grew at 10 μM Hg; all were drastically inhibited at 5 μM Hg; and all but one (Tf-2) grew fairly well at 1 μM Hg in 7 days. Adaptation was again difficult and repeated transfers sometimes resulted in reduced growth after 7 days (Table 8). With a

longer incubation perid (Table 9) Tt, Tf-1, Tf-2, SM-6, SM-7 and SM-8 grew at 5 μM Hg with longer lag periods and generation times. Among 4 strains (SM-1, SM-3, SM-4, SM-5) which grew only at 1 μM Hg, SM-3 and SM-5 grew at 5 μM Hg after adaptation. Most strains adapted to 5 μM Hg grew better (shorter lag period and generation time) than control culture at 5 μM Hg. Thus adaptation was successful.

The sulfur-oxidizing activity was more strongly inhibited by Hg, than Ag, 50% inhibition at 2-5 μM Hg (Table 10). Among control cells SM-1 and SM-6 were the most tolerant, followed by SM-3, SM-5 and SM-8, then by Tt and SM-7. Tf-1, Tf-2 and SM-4 were the most sensitive strains. After adaptation Tf-1, Tf-2, SM-4, SM-7 and SM-8 showed an increased level of tolerance, making the adapted SM-7 and SM-8 as Hg tolerant as the most tolerant strain, SM-6. SM-1 and Tt became more sensitive after adaptation.

The level of Hg concentration inhibiting the sulfur-oxidizing activity was similar to the tolerance level of Hg for growth experiments (1-5 μM).

Effect of Mo and adaptation to Mo

All the strains were more resistant to Mo than to Ag or Hg as shown in Tables 11 and 12. None of the strains grew at 500 μM Mo, but all except SM-3 grew eventually at 100 μM Mo. At 50 μM Mo all strains grew fairly well, although lag periods (e.g. Tf-2 and SM-7) and generation times (e.g. Tf-2) sometimes increased. At lower concentrations Mo stimulated the growth of most strains in 7 days (Table 11) and extended the growth periods from the normal 7 days. SM-4, SM-6, SM-7 and SM-8 were the most tolerant strains and grew at 100 μM Mo essentially with the same generation times as the control culture without Mo, although the lag periods were slightly longer. Tt, Tf-1, Tf-2, SM-1, and SM-5 were not as tolerant with extended lag periods and generation times at 100 μM Mo. SM-3 was the most sensitive and grew only at 50 μM Mo.

Adaptation to 100 μM Mo generally led to better growth in 7 days (Table 11) and shorter generation times and lag periods (Table 12). SM-3 failed to adapt to 100 μM Mo, but adapted well to 50 μM. Mo-adapted cultures grown with Mo normally yielded better final growth yields (Table 13) and all the cultures grown with 100 μM Na_2MoO_4 produced blue cells.

The sulfur-oxidizing activity generally increased in the cells grown with 100 μM Mo (Table 13). Only moderate degrees of inhibition of activity were observed in control cells with 1 mM Mo (8-53%), which decreased at 5 or 10 mM Mo. Of control cells SM-8 was the most tolerant of Mo, followed by SM-1, SM-5, SM-6 and SM-7, by SM-3, by Tf-1 and Tf-2, by Tt, and finally by SM-4. The tolerance increased in all strains adapted to 100 μM Mo. Although not shown in the table adaptation to lower Mo concentrations generally did not increase the sulfur-oxidizing activity or the Mo tolerance of cells.

Effect of As and adaptation to As

Sodium arsenite was less toxic to the growth of various strains than Ag or Hg, but more toxic than Mo (Tables 14 and 15). All the strains except SM-5 grew in the presence of 50 μM As with extended lag periods and generation times. None grew in 100 μM As. At 10 μM As all grew fairly well with somewhat longer lag periods and generation times, except SM-3 and SM-5 whose generation times became 3-4 fold longer.

Tt, Tf-2, SM-1, SM-3, SM-4, SM-6 and SM-7 adapted to 50 μM As successfully with shortening of lag periods and generation times. Tf-1, SM-5 and SM-8 adapted only to 5 or 10 μM As.

The sulfur-oxidizing activity of the control cells was inhibited by 50% in 5-10 mM As (Table 16). The cells adapted to and grown with As had weaker (sometimes much weaker) sulfur-oxidizing activities which were more sensitive to As inhibition (50% inhibition by 1 mM As). Although not shown SM-7 adapted

to 50 μM As produced cells which had only 10% of the sulfur-oxidizing activity of the control cells.

Growth of various strains was totally inhibited at 100 μM (0.1 mM) As, while the inhibition of sulfur oxidation required 1 mM As for 50% inhibition of sensitive As-grown cells. Therefore it is possible that AsO_2^- is inhibiting some other reactions essential for growth (e.g. pyruvate dehydrogenase system).

II. Ferrous iron grown cells

Adaptation to Cu

Two laboratory strains (Tf-1, Tf-2) and six mine isolates (SM-1, SM-2, SM-3, SM-4, SM-5 and SM-8) were used for adaptation to growth in 50 mM $CuSO_4$ with $FeSO_4$ as energy source (Table 17). All except SM-2 grew in the medium with Cu although the lag periods and generation times were extended considerably. Upon three transfers in the Cu-containing medium they were gradually shortened in most cases to similar lengths as those of control cultures without Cu, indicating successful adaptation. The Cu-adapted SM-8 grew faster (shorter lag period, generation time and growth time) than the control.

The ferrous iron oxidation by these cells and the effect by Cu are shown in Table 18. The laboratory strains, Tf-1 and Tf-2, had higher K_m values for Fe^{2+} than mine isolates except SM-2. The Cu-adapted Tf-1 and Tf-2 showed even higher K_m values of 3 mM Fe^{2+} at high Fe^{2+} concentrations. Mine isolates on the other hand generally maintained the low K_m values or lowered them further to 0.2 mM upon adaptatoin to Cu, suggesting their ability to oxidize Fe^{2+} at low concentrations more effectively than laboratory strains.

The inhibition by Cu of iron oxidation by control cells was all competitive at high Fe^{2+} concentrations, but uncompetitive, noncompetitive or competitive

at low Fe^{2+}. Upon Cu adaptation the inhibition pattern remained relatively unchanged in Tf-1 and Tf-2, but changed dramatically in mine isolates. Activation of Fe^{2+} oxidation by Cu was observed in SM-1, SM-3, SM-4 and SM-5 and noncompetitive inhibition at low Fe^{2+} became competitive (activation in SM-3). Activation was more at 83 mM than 167 mM Cu, suggesting an inhibitory action by very high Cu concentrations.

Table 19 shows the effect of Zn^{2+} and Fe^{3+} on the activity of these cells. Control cells were inhibited by Zn competititvely at high Fe^{2+} and competitively, noncompetitively or uncompetitively at low Fe^{2+} concentrations. Fe^{3+} was a competititve inhibitor at all Fe^{2+} concentrations. The Cu-adapted cells of SM-1, SM-3, SM-4 and SM-5 were again activated in their Fe^{2+} oxidation by Zn, more at 83 mM than at 167 mM Zn, similar to Cu effect. The noncompetitive pattern of Zn inhibition at low Fe^{2+} again changed to competitive (activation in SM-3). The inhibition by Fe^{3+} remained competitive after Cu adaptation, except in SM-4 and SM-5 where Fe^{3+} activated the Fe^{2+} oxidation at low Fe^{2+} (0.83 mM Fe^{3+} more activating than 2.5 or 8.3 mM Fe^{3+}).

The most striking feature of Cu adaptation was that many mine strains developed dependency on the presence of metals (Cu^{2+}, Zn^{2+} or Fe^{3+}) for the activation of Fe^{2+} oxidation either at high Fe^{2+} ocnetrations only or at both low and high Fe^{2+} concentrations. Thus the activation was observed in Cu-adapted cells of SM-1 (Cu, Zn), SM-3 (Cu, Zn), SM-4 (Cu, Zn, Fe^{3+} at high Fe^{2+}) and SM-5 (Cu, Zn, Fe^{3+} at high Fe^{2+}). This ability to use metals to increase the ferrous iron oxidation rate may be an important feature of mine isolates in successful leaching of sulfide ores.

Adaptation to Zn

All the strains tested except SM-1 grew in the ferrous iron medium in the presence of 150 mM $ZnSO_4$ with somewhat longer growth times than controls as

shown in Table 20. SM-1 grew in another experiment in 150 mM $ZnSO_4$, but stopped growing after 2 transfers. All the other strains continued to grow at least for 3 transfers often with shortening lag periods or generation times, indicating successful adaptation.

The effect of Zn on the ferrous iron oxidizing activity of these cells is shown in Table 21. The high K_m values for Fe^{2+} of Tf-1, Tf-2 and SM-2 were maintained after Zn adaptation and the low K_m values for Fe^{2+} of other mine isolates became even lower in most cases after Zn adaptation, similarly to Cu adaptation.

The inhibition by Zn of iron oxidation by control cells was all competitive at high Fe^{2+} concentrations, but noncompetitive, competitive or uncompetitive at low Fe^{2+} concentrations. After adaptation to Zn activation of the iron oxidizing activity was observed with SM-3, SM-4 and SM-8 cells and much reduced inhibition was observed with SM-5 cells. So these mine isolates became well adapted to iron oxidation in the presence of Zn.

The ferrous iron oxidation by Zn-adapted cells was activated also by Cu in SM-3, SM-4 and SM-8 and by Fe^{3+} in SM-3 and SM-4 as shown in Table 22. SM-5 cells became also very tolerant of Zn (3 fold increase in K_i values). Thus Zn adaptation increased the Fe^{2+} oxidizing activity in the presence of metals just like Cu adaptation. Zn and Cu activated the iron oxidation in Zn-adapted SM-3, SM-4, and SM-8, while Fe^{3+} activated SM-3 and SM-4, all at high Fe^{2+} concentrations. SM-5 became much more tolerant of both Cu and Zn.

Effect of Ag and adaptation to Ag

All the strains were sensitive to $AgNO_3$ and their growth was inhibited at micromolar concentrations (Table 23). Only Tf-1, SM-3 and SM-4 grew in 1 μM Ag after repeated transfers. SM-2 grew only once in 1 μM Ag, but failed to adapt even to 0.1 μM Ag. SM-5 required a gradual increase of Ag from 0.01 μM to 1 μM

Ag for adaptation. Tf-2 and SM-8 grew and adapted to 0.1 μM Ag, but SM-1 failed to grow in 0.1 μM Ag. Ag prolonged the lag period and generation time of these bacteria, but after adaptation they were usually shortened, sometimes to the levels of control cultures (e.g. Tf-2, SM-8). Generally speaking adaptation was more successful at 0.1 μM Ag. At 1 μM Ag SM-3 grew more slowly after repeated transfers in the Ag-containing medium compared to the initial growth. SM-5 grew in 1 μM Ag only after gradual adaptation from 0.01 μM Ag (3 transfers in 0.01 μM, 1 transfer in 0.1 μM and 3 transfers in 1 μM).

The iron oxidizing activity was inhibited by Ag largely uncompetitively (intercept change in double reciprocal Fe^{2+}-activity plots) with K_i values of 125-525 μM Ag for control cells and 30-260 μM Ag for Ag-adapted cells (Table 24). Thus Ag^{+}, unlike Cu^{2+}, Zn^{2+} or Fe^{3+}, was not competing with the substrate, Fe^{2+}, and the inhibitory concentration was much higher than that for the growth inhibition (0.1-1 μM Ag). The inhibition was generally stronger after adaptation in 1 μM Ag (e.g. Tf-1). The inhibition of iron oxidation was probably much stronger under growth conditions since the oxidation rate in an Oxygraph sometimes slowed down after a linear portion even when the Fe^{2+} concentration was high. This time-dependent inhibition could have contributed to the extreme sensitivity to Ag during growth, but it is also possible that Ag was inhibiting the growth by inhibiting some other reactions necessary for growth.

Effect of Hg and adaptation to Hg

Hg was strongly inhibitory to the growth of all strains at similar concentrations as Ag (Table 25). Only SM-4 and SM-8 grew at 1 μM Hg and the other strains grew only at 0.1 μM Hg. In all cases growth time increased with Hg, but in most cases it was shortened again somewhat after adaptation with the exception of SM-3 and SM-4.

The iron oxidation by control cells was inhibited by Hg uncompetitively with respect to Fe^{2+} (Table 26) with K_i values of 50-210 µM Hg. Therefore Hg was more inhibitory than Ag. Upon adaptation the inhibition remained uncompetitive and the K_i values became centered in a narrow low range (50-80 µM Hg). Thus in many cases Hg became more strongly inhibitory in Hg-grown cells.

Ag and Hg were both strong inhibitors of growth at similar concentrations (0.1-1 µM). Although the inhibition of activity was stronger with Hg than with Ag, both metals were generally uncompetitive inhibitors, more inhibitory with metal-grown cells and sometimes showed time-dependent inhibition. According to Imai et al. (11) and Sugio et al. (12) both Ag and Hg inhibit cytochrome oxidase of the ferrous iron oxidation system. Therefore the similarity observed is in agreement with these reports.

SM-4 is considered as the most tolerant to both Ag and Hg since it is the only strain that grew in 1 µM $AgNO_3$ and in 1 µM $HgCl_2$ successively.

Effect of Mo and adaptation to Mo

Table 27 shows the effect of Mo on the growth of various strains. Mo was not as strongly inhibitory as Ag or Hg, but more inhibitory than Cu or Zn. All strains grew in 0.1 or 1 mM Mo. Thus Tf-2, SM-2, SM-3, SM-4, SM-5 grew at 1 mM Mo and Tf-1, SM-1 and SM-8 grew at 0.1 mM Mo. Only SM-2 and to some degree SM-5 failed to adapt to Mo successfully. There was no stimulation of growth or increased activity by Mo adaptation with iron grown cells unlike those with 0.1 mM Mo-adapted sulfur grown cells (Table 13).

There was a possibility of interaction between Mo and medium components. The M9K medium used contained 0.1 g K_2HPO_4 (0.57 mM) and 120 mM $FeSO_4$. Na_2MoO_4 at 1 mM produced a faint green color and at 10 mM a darker green color in the medium, presumably due to phosphomolybdate (yellow) which was partially reduced by Fe^{2+} to "molybdenum blue". This could have reduced the effective

concentration of Mo as inhibitor.

The iron oxidation was often more strongly inhibited by Mo than the growth with the exception of SM-3 which was highly tolerant (Table 28). The inhibition was generally uncompetitive with or without adaptation and produced complicated patterns of K_i values depending on the concentrations of both Fe^{2+} and Mo. The values ranged from 2 μM (SM-5) to 11.5 mM (SM-3). The effect of adaptation was also complex, some becoming more sensitive (Tf-1, SM-1, SM-4) and others becoming more tolerant (SM-5 and SM-8). Many showed unproportionately high degrees of inhibition when Mo concentration was raised above 100 μM. Some of these complex results may have been due to the interaction between Mo and Fe^{2+}. SM-3 was by far the most tolerant of Mo with K_i value of 11.5 mM which increased further upon adaptation.

The fact that the iron oxidation was much more sensitive than growth was to Mo inhibition in many strains (e.g. SM-5) suggests that the interaction of Mo with phosphate and high Fe^{2+} in the growth medium may have reduced the effective inhibitory concentration of Mo below 0.1 mM or 1 mM used.

Effect of As and adaptation to As

Arsenite at high concentrations precipitated in the Fe^{2+} medium and therefore could not be tested above 1 mM. Growth was little affected by 1 mM As with or without adaptation (Table 29).

Iron oxidation was affected by As by a complex manner (Table 30) perhaps due to some interaction between arsenite and ferrous iron. At low As (normally below 8.3 mM) the inhibition was generally uncompetitive, but at high As (normally 16.7 mM) the pattern changed to competitive (SM-3, SM-4 and SM-5) or no or reduced inhibition (Tf-1, Tf-2, SM-1, SM-2 and SM-8). Even the second group often showed a competitive pattern at high Fe^{2+} concentrations converging towards the control line near the I/V axis, suggesting a possible interaction

between As and Fe^{2+}.

At low As SM-1, SM-2, SM-3, SM-4 and SM-8 were all very tolerant of As (20-40 mM K_i) and SM-5 became very tolerant after adaptation (4->40 mM K_i). The laboratory strains were less tolerant. Adaptation had generally small effect on the inhibition.

There was sometimes time dependent inhibition by As with the levelling off of oxygen uptake rate before Fe^{2+} depletion, similar to Ag or Hg inhibition. This delayed inhibition and uncompetitive nature of inhibition may suggest the inhibition mechanism similar to Ag or Hg.

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Table 1. Inhibitory effect of $CuSO_4$ on the growth on sulfur for 7 days at 25°C of various strains with or without adaptation.

Strains	Growth (% of control culture grown without Cu in mg protein/L)			
	Control culture		Cu-adapted culture	
	$CuSO_4$ concentration			
	50 mM	100 mM	50 mM	100 mM
Tt	10	9.1	93	16
SM-4	20	1.4	36	81
SM-6	4.3	4.3	148	33
SM-7	11	2.1	41	93

All 4 strains adapted well to growth in $CuSO_4$.

Table 2. Effect of $CuSO_4$ on the growth of selected Cu-tolerant strains on sulfur at 25° with or without adaptation.

Strains	Lag period (Lp) in days and generation time (Gt) in hours				
	Control culture			Cu-adapted culture*	
	$CuSO_4$ concentration				
	0 mM	50 mM	100 mM	50 mM	100 mM
Tt (Lp)	3	> 7	> 7	2	3
(Gt)	9			12	26
SM-4 (Lp)	3	3	>10	3	3
(Gt)	8	16		27	10
SM-6 (Lp)	3	> 7	> 7	3	3
(Gt)	9			11	17
SM-7 (Lp)	3	>10	>10	3	4
(Gt)	9			15	9

*Adapted and maintained for 5 months at 50 or 100 mM $CuSO_4$ at 25°C (five or more transfers with 3% inoculum.
">" sign indicates no growth in days.
All 4 strains adapted well to growth in $CuSO_4$.

Table 3. Growth yield and color of packed cells of control and Cu-adapted cells grown for 10 days at 25°C on sulfur with and without $CuSO_4$.

Stains and cells	$CuSO_4$ mM Adaptation	$CuSO_4$ mM Growth	Growth yield mg wet cells per L	Color of packed cells
Tt-0-0	0	0	250	milky white
Tt-Cu50-0	50	0	100	yellowish grey
Tt-Cu50-50	50	50	200	yellowish black
Tt-Cu100-0	100	0	20	yellowish grey
Tt-Cu100-100	100	100	50	yellowish black
SM-4-0-0	0	0	500	milky white
SM-4-Cu50-0	50	0	120	milky grey
SM-4-Cu50-50	50	50	120	grey
SM-4-Cu100-0	100	0	60	milky grey
SM-4-Cu100-100	100	100	340	black
SM-6-0-0	0	0	250	milky yellow
SM-6-Cu50-0	50	0	50	milky yellow
SM-6-Cu50-50	50	50	270	yellowish black
SM-6-Cu100-0	100	0	50	milky yellow
SM-6-Cu-100-100	100	100	50	yellowish green black
SM-7-0-0	0	0	440	faintly brown white
SM-7-Cu50-0	50	0	160	faint grey
SM-7-Cu50-50	50	50	200	greenish grey
SM-7-Cu100-0	100	0	100	faint grey
SM-7-Cu-100-100	100	100	360	brown grey

Most Cu-adapted cultures became dependent on $CuSO_4$ for good growth and produced grey or black cells (darker when grown with $CuSO_4$) due probably to copper sulfide.

Table 4. Sulfur-oxidizing activity of control and Cu-adapted cells grown for 10 days at 25°C on sulfur and effect of $CuSO_4$.

Cells	Activity (nmol O_2/min) per mg wet cells	Activity (nmol O_2/min) per L	Activity (% of control) in 167 mM $CuSO_4$
Tt-0-0	16.8	4,200	64
Tt-Cu50-0	5.6	560	71
Tt-Cu50-50	12.4	2,480	132*
Tt-Cu100-0	16.0	320	140*
Tt-Cu100-100	12.4	620	135*
SM-4-0-0	6.2	3,100	48
SM-4-Cu50-0	2.7	320	60
SM-4-Cu50-50	4.2	500	90
SM-4-Cu100-0	2.7	160	60
SM-4-Cu100-100	3.8	1,290	92
SM-6-0-0	12.8	3,200	50
SM-6-Cu50-0	3.6	180	89
SM-6-Cu50-50	2.4	648	367*
SM-6-Cu100-0	3.0	152	84
SM-6-Cu100-100	8.8	440	210*
SM-7-0-0	8.0	3,520	48
SM-7-Cu50-0	2.0	326	60
SM-7-Cu50-50	2.6	520	90
SM-7-Cu100-0	6.4	640	60
SM-7-Cu100-100	11.2	4,032	92

*Activity higher in the presence of 167 mM $CuSO_4$. All adapted cells become more tolerant of $CuSO_4$ and some dependent on $CuSO_4$ for high activity.

Table 5. Inhibitory effect of $AgNO_3$ on the growth on sulfur for 7 days at 25°C of various strains with or without adaptation.

Strains	Growth (% of control culture grown without Ag in mg protein/L)					
	Control culture				Ag-adapted culture	
	$AgNO_3$ concentration					
	1 µM	5 µM	10 µM	50 µM	1 µM	5 µM
Tt	101	9	6	5	3*	7
Tf-1	50	2	2		46	2
Tf-2	8	4	2			5
SM-1	87	8	1		67	1*
SM-3	98	4	2		5*	3
SM-4	46	4	3	2	43	1
SM-5	84	2	1		73	2
SM-6	104	5	6	6		4
SM-7	91	4	2			6
SM-8	115	9	2			85**

*Large decrease in growth after repeated transfer (adaptation procedure) in Ag-containing media.
**Large increase in growth after adaptation.

Table 6. Effect of $AgNO_3$ on the growth of various strains on sulfur at 25°C with or without adaptation.

Strains	Lag period (Lp) in days and generation time (Gt) in hours						
	Control culture					Ag-adapted culture*	
	$AgNO_3$ concentration						
	0 µM	1 µM	5 µM	10 µM	50 µM	1 µM	5 µM
Tt (Lp)	3	3	14	14	16		10
(Gt)	8	10	16	18	16		20
Tf-1 (Lp)	2	5	>14	>14		5	>14
(Gt)	10	10				10	
Tf-2 (Lp)	3	9	14	>21			13
(Gt)	9	10	23				10
SM-1 (Lp)	3	3	> 7	>10		3	>10
(Gt)	9	8				9	
SM-3 (Lp)	2	2	14	>21			12
(Gt)	10	11	24				20
SM-4 (Lp)	3	6	9	10	>14	6	>14
(Gt)	8	9	15	39		16	
SM-5 (Lp)	2	3	>10	>10		3	>10
(Gt)	7	9				9	
SM-6 (Lp)	3	3	14	16	>21		13
(Gt)	9	9	24	34			12
SM-7 (Lp)	3	3	11	>14			11
(Gt)	9	11	13				15
SM-8 (Lp)	2	2	7	>14			4
(Gt)	9	9	11				13

*Adapted and maintained at 1 µM or 5 µM $AgNO_3$ for 2 or more transfers at 25°C. At higher concentrations of Ag the culture failed to grow after 1 or 2 transfers.

Tolerance

Growth at 10 µM: Tt, SM-4, SM-6; 5 µM: Tf-2, SM-3, SM-7, SM-8;
1 µM: Tf-1, SM-1, SM-5

Adaptation at 5 µM: Tt, Tf-2, SM-3, SM-6, SM-7, SM-8
1 µM: Tf-1, SM-1, SM-4, SM-5

Table 7. Effect of $AgNO_3$ on the sulfur-oxidizing activity of control and Ag-adapted cells grown at 25°C on sulfur.

Strains and cells	Growth time (days)	Growth yield mg wet cells per L	Activity (nmol O_2/min) per L	Activity (% of control)** in $AgNO_3$ (μM) 10	25	50	100	250	500
Tt	7	220	6,336	94	82	71	63	43	31
Tt-Ag5μM*	14	300	9,600	83	70	55	48	34	27
Tf-1	7	250	5,800	90	55		31	22	
Tf-1-Ag1μM*	12	280	5,824		67	58	46	30	
Tf-2	7	200	6,000	80	52		34	26	
Tf-2-Ag5μM	21	500	7,800		69		43	31	
SM-1	7	500	19,200	98	85		63	46	
SM-1-Ag1μM	10	500	14,800		80		53	35	
SM-3	7	230	3,312	87	74	58	47	31	
SM-3-Ag5μM	21	480	6,336		59		36	25	
SM-4	7	330	13,200	91		53	44		22
SM-4-Ag1μM	12	340	11,152	87	71	52	43		20
SM-5	7	400	14,560	91	70		46	31	
SM-5-Ag1μM	10	360	6,192	91	53		41		
SM-6	7	250	5,700	95	82		67	39	
SM-6-Ag5μM	21	350	11,200		75		50	33	
SM-7	7	370	8,880	95	71	60	42		25
SM-7-Ag5μM	21	400	13,440		89		77	62	46
SM-8	7	180	7,200		86		72	54	46
SM-8-Ag5μM	10	200	8,000	62	44		30	20	

*Adapted and grown at 5 μM or 1 μM $AgNO_3$.
**Activity often decreased with time in the presence of $AgNO_3$, e.g. 74 -> 20%, 47 -> 11% after 10 min of reaction. The value given is the initial velocity.

Tolerance of control cells to $AgNO_3$
SM-8 > SM-1 = Tt = SM-6 > SM-3 = SM-5 = SM-7 = SM-4 > Tf-1 = Tf-2

Tolerance after adaptation
Increase: Tf-1, Tf-2, SM-7
No change: SM-4
Decrease: Tt, SM-1, SM-3, SM-5, SM-6, SM-8

Table 8. Inhibitory effect of $HgCl_2$ on the growth on sulfur for 7 days at 25°C of various strains with or without adaptation.

Strains	Growth (% of control culture grown without Hg in mg protein/L					
	Control culture				Hg-adapted culture	
	$HgCl_2$ concentration					
	1 µM	5 µM	10 µM	50 µM	1 µM	5 µM
Tt	54	6	2	3	7*	6
Tf-1	80	11	2		10*	5*
Tf-2	9	5	3		6	2*
SM-1	53	5	1		48	1*
SM-3	95	4	4		3*	1*
SM-4	48	3	4		56	3
SM-5	61	4	2		90**	63**
SM-6	89	4	2	4	4*	4
SM-7	55	5	4			9**
SM-8	112	16	3			56**

*Large decrease in growth after repeated transfers (adaptation procedure) in Hg-containing media.
**Large increase in growth after adaptation.

Table 9. Effect of $HgCl_2$ on the growth of various strains on sulfur at 25°C with or without adaptation.

Strains	Lag period (Lp) in days and generation time (Gt) in hours						
	Control culture					Hg-adapted culture*	
	$HgCl_2$ concentration						
	0 µM	1 µM	5 µM	10 µM	50 µM	1 µM	5 µM
Tt (Lp)	3	3	10	>14	>14		7
(Gt)	8	13	21				11
Tf-1 (Lp)	2	4	6	>14			6
(Gt)	11	13	14				13
Tf-2 (Lp)	3	8	12	>21			12
(Gt)	9	9	21				9
SM-1 (Lp)	3	3	>7	>10		3	>10
(Gt)	9	10				9	
SM-3 (Lp)	2	2	>21	>21			12
(Gt)	10	10					17
SM-4 (Lp)	3	5	>10	>10		5	>14
(Gt)	8	12				13	
SM-5 (Lp)	2	2	>10	>10		2	5
(Gt)	7	10				9	11
SM-6 (Lp)	3	3	10	>10	>10		9
(Gt)	10	11	11				11
SM-7 (Lp)	3	5	10	>14			7
(Gt)	9	10	10				10
SM-8 (Lp)	2	2	7	>14			5
(Gt)	9	9	16				13

*Adapted and maintained at 1 µM or 5 µM $HgCl_2$ for 2 or more transfers at 25°C.

Tolerance Growth at 5 µM: Tt, Tf-1, Tf-2, SM-6, SM-7, SM-8
1 µM: SM-1, SM-3, SM-4, SM-5
Adaptation at 5 µM: Tt, Tf-1, Tf-2, SM-3, SM-5, SM-6, SM-7, SM-8
1 µM: SM-1, SM-4

Table 10. Effect of $HgCl_2$ on the sulfur-oxidizing activity of control and Hg-adapted cells grown at 25°C on sulfur.

Strains and cells	Growth time (days)	Growth yield mg wet cells per L	Activity (nmol O_2/min) per L	Activity (% of control) in $HgCl_2$ (µM) 1.0	2.5	5.0	7.5	10.0
Tt	7	220	6,336	93	64	33	6	7
Tt-Hg5µM*	14	300	11,760	90	47	7		
Tf-1	7	250	5,800		36	22	9	
Tf-1-Hg5µM	14	350	15,400		56	40	3	
Tf-2	7	200	6,000	87	41	19	6	
Tf-2-Hg5µM	21	520	8,736		79	50	12	
SM-1	7	500	19,200		89	54	32	
SM-1-Hg1µM*	10	180	5,112		45	11		
SM-3	7	230	3,312		78	42	14	
SM-3-Hg5µM	21	480	10,368		82	39	3	
SM-4	7	330	13,200	93	42	12		
SM-4-Hg1µM	10	300	13,440		83	49	8	
SM-5	7	400	14,560		86	31	12	
SM-5-Hg5µM	10	350	13,440		75	42	9	
SM-6	7	250	5,700		92	55	26	3
SM-6-Hg5uM	18	500	12,400		93	77	13	3
SM-7	7	370	8,880		65	22	5	
SM-7-Hg5µM	14	420	8,568		92	80	26	6
SM-8	7	180	7,200		84	42	18	2
SM-8-Hg5µM	14	700	20,160		83	58	26	

*Adapted and grown at 5 µM or 1 µM $HgCl_2$

Tolerance of control cells to $HgCl_2$

SM-1 = SM-6 > SM-3 = SM-5 = SM-8 > Tt = SM-7 > Tf-1 = Tf-2 = SM-4

Tolerance after adaptation

Increase: Tf-1, Tf-2, SM-4, SM-7, SM-8

No change: SM-3, SM-5, SM-6

Decrease: Tt, SM-1

Table 11. Inhibitory effect of Na_2MoO_4 on the growth on sulfur for 7 days at 25°C of vairous strains with or without adaptation.

Strains	Growth (% of control culture grown with or without Mo in mg protein/L)								
	Control culture						Mo-adapted culture		
	Na_2MoO_4 concentration								
	1 µM	5 µM	10 µM	50 µM	100 µM	500 µM	5 µM	50 µM	100 µM
Tt	113	108	93	51	4	1	114		27*
Tf-1	108	101	105	94	4	1		91	132*
Tf-2	121	127	124	28	4	3			48*
SM-1	100	95	90	57	4	2		61	45*
SM-3	99	93	85	84	1	2		98	3
SM-4	108	105	92	56	5	2			6
SM-5	112	103	97	76	4	1		79	3
SM-6	96	50	52	44	42	2			44
SM-7	105	114	101	10	4	2			4
SM-8	154	160	163	150	6	3		176	53*

*Large increase in growth after adaptation.

Table 12. Effect of Na_2MoO_4 on the growth of various strains on sulfur at 25°C with or without adaptation.

Strains	Lag period (Lp) in days and generation time (Gt) in hours									
	Control culture							Mo-adapted culture*		
	Na_2MoO_4 concentration									
	0 µM	1 µM	5 µM	10 µM	50 µM	100 µM	500 µM	5 µM	50 µM	100 µM
Tt(Lp)	3	3	3	4	4	10	>18	3		6
(Gt)	8	9	9	12	10	29		8		15
Tf-1(Lp)	3	3	3	3	3	7	>18		3	3
(Gt)	8	9	9	9	9	21			9	8
Tf-2(Lp)	3	3	3	3	7	10	>14			6
(Gt)	9	9	9	10	37	50				9
SM-1(Lp)	3	3	3	3	4	10	>17		4	4
(Gt)	9	10	9	7	10	14			10	10
SM-3(Lp)	2	2	2	3	3	>31	>31		3	>10
(Gt)	9	9	10	10	10				10	
SM-4(Lp)	3	3	3	3	4	8	>12			8
(Gt)	8	8	8	8	8	9				9
SM-5(Lp)	2	2	2	2	2	10	>14		2	10
(Gt)	8	7	7	9	13	19			9	17
SM-6(Lp)	3	3		5	5	5	>14	5		5
(Gt)	9	9		8	9	9		9		8
SM-7(Lp)	3	3	3	3	7	13	>12			13
(Gt)	9	9	8	9	12	10				10
SM-8(Lp)	2	2	2	2	3	13	>18		3	5
(Gt)	9	9	9	9	9	9			10	18

*Adapted and maintained at 5, 50 or 100 µM $NaMoO_4$ for 2 or more transfers at 25°C.

Tolerance Growth at 100 µM: Tt, Tf-1, Tf-2, SM-1, SM-4, SM-5, SM-6, SM-7, SM-8
50 µM: SM-3
Adaptation at 100 µM: Tt, Tf-1, Tf-2, SM-1, SM-4, SM-5, SM-6, SM-7, SM-8
50 µM: SM-3

Table 13. Effect of Na_2MoO_4 on the sulfur-oxidizing activity of control and Mo-adapted cells grown at 25°C on sulfur.

Strains and cells	Growth time (days)	Growth yield mg wet cells per L	Activity (nmol O_2/min) per L	Activity (nmol O_2/min) per mg wet cells	Activity (% of control) in Na_2MoO_4 (mM) 1	5	10
Tt	7	220	6,336	29	64	61	
Tt-Mo 100 µM*	10	132**	9,498	72	93	93	
Tf-1	7	250	5,800	23	70	72	
Tf-1-Mo 100 µM	12	780**	21,840	28	83	83	
Tf-2	7	200	6,000	30	65	75	103
Tf-2-Mo 100 µM	12	580**	25,288	44	89	100	
SM-1	7	500	19,200	38	80	83	
SM-1-Mo 100 µM	10	280**	15,008	54	86	82	
SM-3	7	230	3,312	14	72	78	
SM-3-Mo 50 µM*	12	980	15,680	16	66	75	
SM-4	7	330	13,200	40	47	49	
SM-4-Mo 100 µM	12	300**	15,840	53	80	87	
SM-5	7	400	14,560	36	75	86	
SM-5-Mo 100 µM	14	170**	8,704	51	91	89	
SM-6	7	250	5,700	23	78	86	
SM-6-Mo 100 µM	14	440**	12,496	28	83	87	
SM-7	7	370	8,880	24	77	78	
SM-7-Mo 100 µM	18	360**	22,464	62	87	88	
SM-8	7	180	7,200	40	92	92	102
SM-8-Mo 100 µM	12	240**	7,680	32	95	88	

*Adapted and grown at 100 or 50 µM Na_2MoO_4.
**All cells adapted and grown at 100 µM Na_2MoO_4 were blue, probably due to "molybdenum blue" formation and had high levels of sulfur-oxidizing activity.

Tolerance of control cells to Na_2MoO_4
SM-8 > SM-1 = SM-5 = SM-6 = SM-7 > SM-3 > Tf-1 = Tf-2 > Tt > SM-4
Only moderate inhibition at 1 mM, often less inhibition at higher concentrations.

Tolerance after adaptation
Increase in all strains adapted to 100 µM Na_2MoO_4.

Table 14. Inhibitory effect of $NaAsO_2$ on the growth on sulfur for 7 days at 25°C of various strains with or without adaptation.

Strains	Growth (% of control culture grown without As in mg protein/L)								
	Control culture						As-adapted culture		
	$NaAsO_2$ concentration								
	1 µM	5 µM	10 µM	50 µM	100 µM	500 µM	5 µM	10 µM	50 µM
Tt	106	46	63	4	1		52		42*
Tf-1	87	47	37	3	2			43	
Tf-2	92	79	60	5	1			67	5
SM-1	81	72	35	2	1			38	42*
SM-3	87	79	4	4	1		85	7	6
SM-4	103	52	38	4	1	2	50	40	32*
SM-5	116	102	27	1	2			39	
SM-6	85	67	66	5	2				4
SM-7	55	43	23	5	1	2			10
SM-8	132	97	80	4	3		106		

*Large increase in growth after adaptation.

Table 15. Effect of $NaAsO_2$ on the growth of various strains on sulfur at 25°C with or without adaptation.

Strains	Lag period (Lp) in days and generation time (Gt) in hours								
	Control culture						As-adapted culture*		
	$NaAsO_2$ concentration								
	0 µM	1 µM	5 µM	10 µM	50 µM	100 µM	5 µM	10 µM	50 µM
Tt(Lp)	3	3	4	3	22	>28	7		6
(Gt)	8	9	9	10	13		10		9
Tf-1(Lp)	3	3	4	5	30	>36		5	
(Gt)	8	9	12	11	27			10	
Tf-2(Lp)	3	3	3	4	28	>32		4	7
(Gt)	9	9	10	9	40			11	19
SM-1(Lp)	3	3	3	6	29	>34		6	5
(Gt)	9	9	10	10	12			12	12
SM-3(Lp)	2	2	4	12	28	>33	4	7	10
(Gt)	9	9	15	33	18		10	19	12
SM-4(Lp)	3	3	5	4	25	>30	5	4	6
(Gt)	8	9	9	9	9		11	9	9
SM-5(Lp)	2	2	2	5	>14	>14		5	
(Gt)	8	7	8	25				11	
SM-6(Lp)	3	3		4	21	>28	4		7
(Gt)	9	9		9	40		9		9
SM-7(Lp)	3	5		6	24	>30	5		6
(Gt)	9	9		9	18		10		10
SM-8(Lp)	2	2	2	2	24	>28	2		
(Gt)	9	12	10	10	18		12		

*Adapted and maintained at 5, 10, or 50 µM $NaAsO_2$ for 2 or more transfers at 25°C.

Tolerance
Growth at 50 µM: Tt, Tf-1, Tf-2, SM-1, SM-3, SM-4, SM-6, SM-7, SM-8
10 µM: SM-5
Adaptation at 50 µM: Tt, Tf-2, SM-1, SM-3, SM-4, SM-6, SM-7
at 5 or 10 µM: Tf-1, SM-5, SM-8.

Table 16. Effect of $NaASO_2$ on the sulfur-oxidizing activity of control and As-adapted cells grown at 25°C on sulfur.

Strains and cells	Growth time (days)	Growth yield mg wet cells per L	Activity (nmol O_2/min) per L	Activity (nmol O_2/min) per mg wet cells	Activity (% of control) in $NaAsO_2$ (mM) 1	5	10
Tt	7	220	6,336	29	74	50	
Tt-As50 µM*	14	620	4,464	7	56	44	
Tf-1	7	250	5,800	23	81	44	
Tf-1-As10µM*	12	400	5,920	15	54	37	
Tf-2	7	200	6,000	30	77	49	
Tf-2-As50µM	14	280	1,792	6	50		
SM-1	7	500	19,200	38	91	71	
SM-1-As50µM	14	550	6,600	12	62	45	
SM-3	7	230	3,312	14	76	44	
SM-3-As50µM	14	400	2,080	5	46	37	
SM-4	7	330	13,200	40	84	57	
SM-4-As50µM	14	600	6,720	11	54	36	
SM-5	7	400	14,560	36	86	64	40
SM-5-As10µM	14	310	2,728	9	55	41	
SM-6	7	250	5,700	23	80	56	
SM-6-As50µM	14	300	5,040	17	62	40	
SM-7	7	370	8,880	24	73	48	
SM-7-AS5µM	12	320	3,584	11	59	44	
SM-8	7	180	7,200	40	94	78	56
SM-8-As5µM	12	400	4,960	12	44	39	

*Adapted and grown at 5, 50, or 10 µM $NaAsO_2$.
All cells adapted and grown with $NaAsO_2$ had decreased levels of sulfur-oxidizing activity.

Tolerance of control cells to $NaAsO_2$ (50% inhibition in 5-10 mM As - all strains)
SM-8 > SM-1 > SM-5 = SM-4 = SM-6 > Tf-1 = Tf-2 = SM-7 > SM-3 = Tt
Tolerance after adaptation (50% inhibition in 1 mM As - all strains)
All cells became more sensitive to inhibition by $NaAsO_2$.

Table 17. Effect of $CuSO_4$ on the growth of various strains of ferrous iron at 25°C with or without adaptation.

Strains and cells	$CuSO_4$ Growth mM	Lag period hours	Generation time hours	Growth time hours	Growth yield per L		Activity (V_{max}) nmol O_2/min per mg protein
					mg protein	mg wet cells	
Tf-1	0	0-5	7	15	8.8	83	1,250
Tf-1	50	41	18	85			
Tf-1-Cu50*	50	0-5	9	30	12.0	75	1,075
Tf-2	0	0-5	8	25	15.4	80	1,250
Tf-2	50	65	10	92			
Tf-2-Cu50	50	0-5	10	27	9.6	75	950
SM-1	0	325	20	350	2.6	20	680
SM-1	50	300	75	475			
SM-1-Cu50	50	190	40	290	3.7	25	345
SM-2	0	0-5	19	65	3.3	23	360
SM-2	50	NG**					
SM-3	0	0-5	12	35	7.2	49	230
SM-3	50	20	82	145			
SM-3-Cu50	50	10	25	75	5.7	87	205
SM-4	0	0-5	12	25	3.4	40	1,740
SM-4	50	100	34	150			
SM-4-Cu50	50	0-5	16	46	9.7	54	510
SM-5	0	40	10	70	49.1	332	800
SM-5	50	60	20	105			
SM-5-Cu50	50	0-5	18	50	9.7	110	1,100
SM-8	0	20	30	100	5.0	28	690
SM-8	50	100	56	240			
SM-8-Cu50	50	0-5	17	50	2.0	37	830

*Adapted to growth in 50 mM $CuSO_4$ at 25° for several transfers.
**NG: No growth.

Table 18. Effect of Cu^{2+} on the ferrous iron oxidizing activity of various strains with or without adaptation.

Strains and cells	$CuSO_4$ Growth mM	Km mM Fe^{2+}		Inhibition by Cu^{2+} Type*		Ki (mM)**	
		Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}
Tf-1	0	1.2	1.2	UC	C	50(i)	83(s)
Tf-1-Cu50	50	1.3	3.1	NC	C	135(i)	115(s)
Tf-2	0	1.0	1.0	NC	C	90(i)	130(s)
Tf-2-Cu50	50	0.8	2.9	NC	C	150(i)	70(s)
SM-1	0	0.4	0.4	C	C	54(s)	54(s)
SM-1-Cu50	0	0.2	2.2	A***	NI	A	NI
SM-2	0	1.0	1.0	UC	C	100(i)	130(s)
SM-3	0	0.2	0.6	NC	C	150(i)	70(s)
SM-3-Cu50	50	0.2	1.1	A***	A***	A	A
SM-4	0	0.5	0.5	NC	C	155(i)	30(s)
SM-4-Cu50	50	0.2	0.2	C	A***	90(s)	A
SM-5	0	0.4	0.4	NC	C	75(i)	50(s)
SM-5-Cu50	50	0.3	0.3	C	A***	128(s)	A
SM-8	0	0.3	0.3	NC	C	60(i)	68(s)
SM-8-Cu50	50	0.2	0.2	C	C	32(s)	32(s)

*Type C = competitive, UC = uncompetitive, NC = noncompetitive, NI = no inhibition
Low Fe^{2+}, high Fe^{2+}: Biphasic pattern in double reciprocal plots with one type of inhibition at low Fe^{2+} concentrations and another type at high Fe^{2+} concentrations.

**Ki i = intercept, s = slope; i affected by UC and NC, s affected by C and NC.

***A A = activation by Cu^{2+}, 83 mM Cu^{2+} activating more than 167 mM Cu^{2+}.

Table 19. Effect of Zn^{2+} and Fe^{2+} on the ferrous iron oxidizing activity of various strains with or without copper adaptation.

Strains and cells	$CuSO_4$ Growth mM	Inhibition by Zn^{2+} Type		Inhibition by Zn^{2+} Ki (mM)		Inhibition by Fe^{3+} Type		Inhibition by Fe^{3+} Ki (mM)	
		Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}
Tf-1	0	UC	C	80(i)	75(i)	C	C	1.5(s)	1.5(s)
Tf-1-Cu50	50	NI	NI	NI	NI	C	C	4.3(s)	2.7(s)
Tf-2	0	C	C	140(s)	140(s)	C	C	1.8(s)	1.8(s)
Tf-2-Cu50	50	C	C	130(s)	170(s)	C	C	1.5(s)	2.4(s)
SM-1	0	C	C	77(s)	77(s)	C	C	1.9(s)	1.9(s)
SM-1-Cu50	50	A*	A*	A	A	C	C	7.0(s)	7.0(s)
SM-2	0	C	C	300(s)	300(s)	C	C	2.0(s)	2.0(s)
SM-3	0	NC	C	180(i)	180(s)	C	C	1.2(s)	10.0(s)
SM-3-Cu50	50	A**	A**	A	A	C	C	2.1(s)	2.2(s)
SM-4	0	NC	C	215(i)	46(s)	C	C	2.1(s)	2.1(s)
SM-4-Cu50	50	C	A*	125(s)	A	C	A*	2.6(s)	A
SM-5	0	NC	C	150(i)	55(s)	C	C	1.4(s)	1.4(s)
SM-5-Cu50	50	C	A*	85(s)	A	C	A*	1.0(s)	
SM-8	0	NC	C	120(i)	116(s)	C	C	3.2(s)	3.2(s)
SM-8-Cu50	50	C	C	60(s)	60(s)	C	C	0.6(s)	0.6(s)

*Activation by Zn^{2+} or Fe^{3+} (0.83-8.3 mM), lower concentrations activating more.
**Activation by Zn^{2+} 167 mM Zn^{2+} activating more than 83 mM Zn^{2+}.

Table 20. Effect of $ZnSO_4$ on the growth of various strains on ferrous iron at 25°C with or without adaptation.

Strains and cells	$ZnSO_4$ Growth mM	Lag period hours	Generation time hours	Growth time hours	Growth yield per L		Activity (V_{max}) nmol O_2/min per mg protein
					mg protein	mg wet cells	
Tf-1	0	0-5	7	15	8.8	83	1,250
Tf-1	150	0-5	9	25			
Tf-1-Zn150*	150	0-5	14	40	20.0	170	1,200
Tf-2	0	0-5	8	25	15.4	80	1,250
Tf-2	150	32	9	55			
Tf-2-Zn150	150	0-5	9	30	16.0	125	1,050
SM-1	0	325	20	350	2.6	20	680
SM-1	150	NG**					
SM-2	0	200	20	265	3.3	23	360
SM-2	150	280	30	360			
SM-2-Zn150	150	110	30	210	7.1	50	600
SM-3	0	0-5	12	35	7.2	49	230
SM-3	150	0-5	45	135			
SM-3-Zn150	150	0-5	12	35	5.0	50	280
SM-4	0	0-5	12	25	3.4	40	1,740
SM-4	150	0-5	14	50			
SM-4-Zn150	150	0-10	12	50	14.0	85	670
SM-5	0	40	10	70	49.1	332	800
SM-5	150	80	10	105			
SM-5-Zn150	150	20	10	45	24.2	220	750
SM-8	0	30	20	100	5.0	28	690
SM-8	150	60	30	150			
SM-8-Zn150	150	0-5	17	50	8.6	50	115

*Adapted to growth in 150 mM $ZnSO_4$ at 25°C for several transfers.
**NG: No growth.

Table 21. Effect of Zn^{2+} on the ferrous iron oxidizing activity of various strains with or without adaptation.

Strains and cells	$ZnSO_4$ Growth mM	K_m mM Fe^{2+}		Inhibition by Zn^{2+} Type		K_i (mM)	
		Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}
Tf-1	0	1.2	1.2	UC	C	80(i)	75(s)
Tf-1-Zn150	150	0.6	4.6	NC	C	400(i)	150(s)
Tf-2	0	1.0	1.0	C	C	140(s)	140(s)
Tf-2-Zn150	150	0.9	0.9	NC	C	100(i)	160(s)
SM-1	0	0.4	0.4	C	C	77(s)	77(s)
SM-2	0	1.0	1.0	C	C	300(s)	300(s)
SM-2-Zn150	150	1.7	1.7	C	C	170(s)	170(s)
SM-3	0	0.2	0.6	NC	C	180(i)	180(s)
SM-3-Zn150	150	0.3	0.3	NC	A*	600(i)	A
SM-4	0	0.5	0.5	NC	C	215(i)	46(s)
SM-4-Zn150	150	0.3	0.3	C	A*	130(s)	A
SM-5	0	0.4	0.4	NC	C	160(i)	55(s)
SM-5-Zn150	150	0.3	1.1	NC	C	500(i)	325(s)
SM-8	0	0.3	0.3	NC	C	120(i)	120(s)
SM-8-Zn150	150	0.1	0.1	C	A*	70(s)	A

*Activation by Zn^{2+} both at 83 mM and 167 mM.

Table 22. Effect of Cu^{2+} and Fe^{3+} on the ferrous iron oxidizing activity of various strains with or without zinc adaptation.

Strains and cells	$ZnSO_4$ Growth mM	Inhibition by Cu^{2+}: Type		Inhibition by Cu^{2+}: K_i (mM)		Inhibition by Fe^{3+}: Type		Inhibition by Fe^{3+}: K_i (mM)	
		Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}	Low Fe^{2+}	High Fe^{2+}
Tf-1	0	UC	C	50(i)	83(s)	C	C	1.5(s)	1.5(s)
Tf-1-Zn150	150	NC	C	185(i)	105(s)	NC	C	1.6(i)	4.0(s)
Tf-2	0	NC	C	90(i)	130(s)	C	C	1.8(s)	1.8(s)
Tf-2-Zn150	150	NC	C	65(i)	130(s)	NC	C	4.0(i)	4.6(s)
SM-1	0	C	C	54(s)	54(s)	C	C	1.9(s)	1.9(s)
SM-2	0	UC	C	100(i)	130(s)	C	C	2.0(s)	2.5(s)
SM-2-Zn150	150	UC	C	80(i)	70(s)	C	C	3.5(s)	3.5(s)
SM-3	0	NC	C	150(i)	70(s)	C	C	1.2(s)	10.0(s)
SM-3-Zn150	150	NC	A*	900(i)	A	C	A*	4.5(s)	A
SM-4	0	NC	C	155(i)	30(s)	C	C	2.1(s)	2.1(s)
SM-4-Zn150	150	C	A*	80(i)	A	C	A*	2.0(s)	A
SM-5	0	NC	C	75(i)	50(s)	C	C	1.4(s)	1.4(s)
SM-5-Zn150	150	NC	C	250(i)	165(s)	C	C	1.4(s)	1.4)s)
SM-8	0	NC	C	60(i)	68(s)	C	C	3.2(s)	3.2(s)
SM-8-Zn150	150	C	A*	30(s)	A	C	C	0.5(s)	0.5(s)

*Activation by Cu^{2+} (83 and 167 mM) or Fe^{3+} (0.83-8.3 mM), lower concentration activating more.

Table 23. Effect of $AgNO_3$ on the growth of various strains on ferrous iron at 25°C with or without adaptation.

Strains and cells	$AgNO_3$ Growth µM	Lag period hours	Generation time hours	Growth time hours	Growth yield per L		Activity (V_{max}) nmol O_2/min per mg protein
					mg protein	mg wet cells	
Tf-1	0	0-5	10	45	6.0	45	770
Tf-1	1	210	110	600			
Tf-1-Ag1*	1	0-5	20	80	6.1	42	1,430
Tf-2	0	0-5	8	30	8.5	50	1,430
Tf-2	0.1	300	20	380			
Tf-2-Ag0.1*	0.1	0-5	8	30	1.3	13	715
SM-1	0	80	70	200	2.1	22	600
SM-1	0.1	NG**					
SM-2	0	50	8	60	13.8	42	330
SM-2	1	50	8	60			
SM-2-Ag1	0.1	NG**					
SM-3	0	0-5	12	35	3.0	25	670
SM-3	1	15-20	15	60			
SM-3-Ag1	1	15-20	70	190	0.8	23	1,110
SM-4	0	0-5	10	40	5.2	45	770
SM-4	1	160	20	235			
SM-4-Ag1	1	120	25	190	3.2	25	590
SM-5	0	0-5	8	35	4.3	42	670
SM-5	0.01	285	25	410			
SM-5-Ag1	1	20	10	55	9.0	50	385
SM-8	0	0-5	9	27	6.4	53	1,670
SM-8	0.1	0-5	22	100			
SM-8-Ag1	0.1	0-5	9	28	16.8	42	670

*Adapted to growth in 1 or 0.1 µM $AgNO_3$ at 25°C for several transfers, except SM-2 which grew only once in 1 µM Ag^+.
**NG = No growth.

Table 24. Effect of Ag^+ on the ferrous iron oxidizing activity of various strains with or without adaptation.

Strains and cells	$AgNO_3$ Growth µM	Km mM Fe^{2+}	Inhibition by Ag^+ Type	Inhibition by Ag^+ K_i (µM)
Tf-1	0	0.23	UC	250 (i)
Tf-1-Ag1	1	0.36	UC	30 (i)
Tf-2	0	0.34	UC	150 (i)
Tf-2-Ag0.1	0.1	0.20	UC	260 (i)
SM-1	0	0.33	NC	300 (i)
SM-2	0	0.16	NC	220 (i)
SM-3	0	0.17	UC	340 (i)
SM-3-Ag1	1	0.17	NC	175 (i)
SM-4	0	0.38	UC	525 (i)
SM-4-Ag1	1	2.0	NC	250 (i)
SM-5	0	0.36	NC	230 (i)
SM-5-Ag1	1	0.24	NC	180 (i)
SM-8	0	0.25	UC	230 (i)
SM-8-Ag0.1	0.1	0.29	UC	250 (i)

Table 25. Effect of $HgCl_2$ on the growth of various strains on ferrous iron at 25°C with or without adaptation.

Strains and cells	$HgCl_2$ Growth µM	Lag period hours	Generation time hours	Growth time hours	Growth yield per L		Activity (V_{max}) nmol O_2/min per mg protein
					mg protein	mg wet cells	
Tf-1	0	0-5	8	20	6.0	45	770
Tf-1	0.1	0-5	29	90			
Tf-1-Hg0.1*	0.1	0-5	10	35	6.1	42	1,400
Tf-2	0	0-5	9	30	8.5	50	1,430
Tf-2	0.1	80	18	135			
Tf-2-Hg0.1	0.1	0-5	8	28	5.7	48	1,700
SM-1	0	60	8	80	2.1	22	600
SM-1	0.1	120	24	200			
SM-1-Hg0.1	0.1	40	13	75	6.8	64	1,200
SM-2	0	85	23	140			
SM-2	0.1	150	35	240			
SM-2-Hg0.1	0.1	75	35	160	0.3	6	385
SM-3	0	0-5	12	35	3.0	25	670
SM-3	0.1	40	62	150			
SM-3-Hg0.1	0.1	115	28	180	11.0	50	2,200
SM-4	0	0-5	10	40	5.2	45	770
SM-4	1	285	25	350			
SM-4-Hg1*	1	500	25	550	4.0	30	510
SM-5	0	0-5	8	35	4.3	42	670
SM-5	0.1	170	90	325			
SM-5-Hg0.1	0.1	140	12	170	1.8	20	570
SM-8	0	0-5	30	90	6.4	53	1,670
SM-8	1	260	20	310			
SM-8-Hg1	1	0-5	23	80	0.4	13	1,500

*Adapted to growth in 0.1 or 1 µM $HgCl_2$ at 25°C for several transfers.

Table 26. Effect of Hg^{2+} on the ferrous iron oxidizing activity of various strains with or without adaptation.

Strains and cells	$HgCl_2$ Growth µM	K_m mM Fe^{2+}	Inhibition by Hg^{2+} Type	K_i (µM)
Tf-1	0	0.23	UC	50 (i)
Tf-1-Hg0.1	0.1	0.33	UC	50 (i)
Tf-2	0	0.34	UC	130 (i)
Tf-2-Hg0.1	0.1	0.30	UC	70 (i)
SM-1	0	0.33	UC	60 (i)
SM-1-Hg0.1	0.1	0.44	UC	50 (i)
SM-2	0	0.16	UC	50 (i)
SM-2-Hg1	1	0.09	UC	50 (i)
SM-3	0	0.17	UC	210 (i)
SM-Hg0.1	0.1	0.56	UC	60 (i)
SM-4	0	0.38	UC	125 (i)
SM-4-Hg1	1	0.23	UC	50 (i)
SM-5	0	0.36	NC	125 (i)
SM-5-Hg0.1	0.1	0.24	UC	60 (i)
SM-8	0	0.24	UC	65 (i)
SM-8-Hg1	1	0.16	UC	80 (i)

Table 27. Effect of Na_2MoO_4 on the growth of various strains on ferrous iron at 25°C with or without adaptation.

Strains and cells	Na_2MoO_4 Growth mM	Lag period hours	Generation time hours	Growth time hours	Growth yield per L		Activity (V_{max}) nmol O_2/min per mg protein
					mg protein	mg wet cells	
Tf-1	0	0-5	7	22	8.0	87	500
Tf-1	0.1	0-5	8	25			
Tf-1-Mo0.1*	0.1	0-5	8	26	10.0	33	490
Tf-2	0	0-5	7	21	6.0	42	670
Tf-2	1	45	14	82			
Tf-2-Mo1*	1	21	9	52	8.5	50	595
SM-1	0	0-5	7	24	21.6	60	570
SM-1	0.1	0-5	7	25			
SM-1-Mo0.1	0.1	0-5	8	22	4.2	13	345
SM-3	0	0-5	10	32	10.7	93	690
SM-3	1	270	12	295			
SM-3-Mo1	1	0-5	17	50	16.7	167	740
SM-4	0	0-5	10	38	4.3	27	910
SM-4	1	140	20	200			
SM-4-Mo1	1	45	14	90	4.8	27	625
SM-5	0	0-5	8	30	10.8	100	833
SM-5	1	0-5	8	30			
SM-5-Mo1	1	210	25	280	3.9	44	590
SM-8	0	0-5	9	26	6.4	53	1670
SM-8	0.1	23	9	47			
SM-8-Mo0.1	0.1	0-5	8	24	4.5	53	1540

*Adapted to growth in 0.1 or 1 mM Na_2MoO_4 at 25°C for several transfers. SM-2 grew twice in 1 mM Na_2MoO_4 but stopped growing in the third transfer.

Table 28. Effect of MoO_4^{2-} on the ferrous iron oxidizing activity of various strains with or without adaptation.

Strains and cells	Na_2MoO_4 Growth mM	Km mM Fe^{2+}	Inhibition by MoO_4^{2-} Type		K_i (mM)*	
			Low Mo	High Mo	Low Mo	High Mo
Tf-1	0	0.40	NI	NC	NI	0.08(i)**
Tf-1-Mo0.1	0.1	0.40	UC(C)	UC(C)	0.28(i)(0.08(s))	0.15(i)(0.01(s))**
Tf-2	0	0.36	NC(C)	NC(C)	0.09(i)(0.01(s))	0.09(i)(0.01(s))
Tf-2-Mo1	1	0.40	C	C	0.09(s)	0.09(s)
SM-1	0	0.19	UC	UC	0.60(i)	1.3(i)
SM-1-Mo0.1	0.1	0.27	C	UC	0.15(s)	0.03(i)**
SM-2	0	0.16	UC	UC	0.24(i)	0.08(i)**
SM-3	0	0.12	UC	UC	11.5(i)	11.5(i)
SM-3-Mo1	1	0.08	UC	UC	10.5(i)	45 (i)
SM-4	0	0.14	UC	NC	0.16(i)	0.05(i)**
SM-4-Mo1	1	0.13	UC	UC	0.09(i)	0.06(i)
SM-5	0	0.30	C	NC	0.002(s)	0.005(s)
SM-5-Mo1	1	0.15	UC	UC	0.13 (i)	0.02 (i)**
SM-8	0	0.24	UC	NC	0.18 (i)	0.06 (i)**
SM-8-Mo	0.1	0.23	UC	UC	1.2 (i)	0.05 (i)**

*Type of inhibition was often affected by the concentration of MoO_4^{2-} and sometimes (Tf-1-Mo and Tf-2) by Fe^{2+} concentrations (high Fe^{2+} in brackets) leading to different K_i values.
**Unproportionately high degree of inhibition when MoO_4^{2-} concentration was raised.
SM-3: most tolerant. SM-5: most sensitive (only before adaptation).

Table 29. Effect of $NaAsO_2$ on the growth of various strains on ferrous iron at 25°C with or without adaptation.

Strains and cells	$NaAsO_2$ Growth mM	Lag period hours	Generation time hours	Growth time hours	Growth yield per L		Activity (V_{max}) nmol O_2/min per mg protein
					mg protein	mg wet cells	
Tf-1	0	0-5	7	21	8.0	87	530
Tf-1	1	0-5	8	26			
Tf-1-Asl*	1	0-5	9	26	19.0	60	640
Tf-2	0	0-5	7	21	6.0	42	670
Tf-2	1	0-5	8	28			
Tf-2-Asl	1	0-5	8	28	32.0	100	750
SM-1	0	0-5	7	24	21.6	60	570
SM-1	1	0-5	9	32			
SM-1-Asl	1	0-5	10	31	12.0	42	445
SM-2	0	50	4	60	13.8	42	330
SM-2	1	50	4	60			
SM-2-Asl	1	5-10	4	20	8.8	25	570
SM-3	0	0-5	11	33	10.7	93	690
SM-3	1	0-5	11	39			
SM-3-Asl	1	0-5	11	34	17.5	120	625
SM-4	0	0-5	11	40	4.3	27	910
SM-4	1	0-5	14	45			
SM-4-Asl	1	5	12	47	12.2	100	1190
SM-5	0	5-10	8	31	10.8	100	830
SM-5	1	0-5	7	25			
SM-5-Asl	1	0-5	7	23	6.1	63	950
SM-8	0	0-5	9	27	6.4	53	1670
SM-8	1	15	9	40			
SM-8-Asl	1	10	9	38	8.2	60	1430

*Adapted to growth in 1 mM $NaAsO_2$ at 25°C for several transfers.

Table 30. Effect of AsO_2^- on the ferrous iron oxidizing activity of various strains with or without adaptation.

Strains and cells	$NaAsO_2$ Growth mM	K_m mM Fe^{2+}	Inhibition by AsO_2^- Type*		Inhibition by AsO_2^- K_i (mM)*	
			Low As	High As	Low As	High As
Tf-1	0	0.16	NC	C	6.0(i)	6.0(s)
Tf-1-As1	1	0.20	C	NI(NC)	2.3(s)	NI (17.5(i))
Tf-2	0	0.36	UC(C)	NI(C)	17.0(i)(4.0(s))	NI (2.0(s))
Tf-2-As1	1	0.36	UC	NI(C)	12.0(i)	NI (1.5(s))
SM-1	0	0.20	UC	UC	37(i)	90(i)
SM-1-As1	1	0.20	NI	NI(C)	NI	NI (10.0(s))
SM-2	0	0.16	UC	UC	40(i)	40(i)
SM-2-As-1	1	0.13	UC	UC	37(i)	37(i)
SM-3	0	0.12	UC	C	60(i)	2.5(s)
SM-3-As1	1	0.28	UC	C	40(i)	2.5(s)
SM-4	0	0.14	UC	C	20(i)	2.5(s)
SM-4-As1	1	0.14	UC	C(UC)	30(i)	2.0(s)(45(i))
SM-5	0	0.30	NC	NC(C)	4.0(i)	4.0(i)(0.5(s))
SM-5-As1	1	0.30	UC	C	40(i)	2.5(s)
SM-8	0	0.24	UC	NI(UC)	23(i)	(45(i))
SM-8-As1	1	0.24	UC	UC	45(i)	45(i)

*Type of inhibitiion was often affected by the concentrations of AsO_2^- and Fe^{2+} concentrations (high Fe^{2+} in brackets) leading to different K_i values.
In some cases there was no inhibition (NI) at 8.3 or 16.7 mM AsO_2^- (high As) particularly at low Fe^{2+} concentrations.

STAGE III

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EXECUTIVE SUMMARY

Shake flask leaching studies of metal extraction (Cu, Zn and Fe) from a complex sulfide ore were carried out using sulfur-grown *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* strains or ferrous iron-grown *T. ferrooxidans* strains. A column leaching study was carried out with four selected strains of bacteria.

With the No. 1 ore SM-6 and SM-7 achieved the best leaching of Cu and Zn among sulfur-grown cells in shake flask experiments, particularly after ore adaptation, followed by ore-adapted strains of Tf-1, Tf-2, SM-1, SM-3, SM-4 (Cu-adapted) and SM-8. Tt and SM-5 leached very little metals and failed to adapt to the ore.

With the No. 3 ore the initial addition of sulfuric acid was essential for the maintenance of low pH, and thus the metal leaching. Tf-2, SM-6, SM-7 and SM-8 effectively leached metals and the ore adaptation improved the performance further. SM-1 leached metals only after ore adaptation and SM-4 only after Cu adaptation. Tt, Tf-1, SM-3, SM-4 and SM-5 cells died during the incubation with the ore. There was a good correlation between the low final pH and the high metal solubilization.

Among the ferrous iron-grown cells SM-4 and SM-5 solubilized Cu and Zn from the No. 1 ore consistently without adaptation. SM-1, SM-2 and SM-8 gave variable results and SM-3 leached metals only after adaptation. Tf-1 and Tf-2 were ineffective toward the No. 1 ore. The bacterial activity determination of collected cells showed bacterial growth during leaching and increased ratios of sulfur-oxidizing activity over Fe^{2+} oxidizing activity.

The metal leaching from the No. 2 ore by the ferrous iron grown cells was not as extensive (only doubling of control values) as that of No. 1 ore and showed little effect of strain differences. The initial addition of acid was

necessary. Cu adaptation generally increased the metal leaching, but not the ore adaptation. Bacterial growth (as measured by the oxidation activities) of all strains on this ore was very good, often exceeding ten times the growth obtained in the Fe^{2+} medium and the sulfur-oxidizing activity to Fe^{2+}-oxidizing activity ratios were much higher.

The column leaching experiments showed effective leaching of No. 2 ore by all four strains tested; sulfur-grown SM-6 and SM-7 and Fe^{2+}-grown Tf-2 and SM-4. Considerable amounts of sulfuric acid were required initially but the pH stabilized later. Interestingly SM-6 and SM-7 cells collected oxidized Fe^{2+} faster than sulfur and became indistinguishable from SM-4 or Tf-2 after 105 days with respect to the sulfur-oxidizing activity to the Fe^{2+}-oxidizing activity ratio.

In conclusion sulfur-grown SM-6 and SM-7 and ferrous iron-grown SM-4 and SM-5 are the best strains for the extraction of Cu and Zn from a sulfide ore studied. Other naturally isolated strains and even laboratory strains have some potential for effective leaching depending on growth substrates (Fe^{2+}, S^0 or ore) and the nature of ore samples.

MATERIALS AND METHODS

Bacterial strains and growth

Thiobacillus thiooxidans ATCC 8085 (Tt), *Thiobacillus ferrooxidans* ATCC 13661 (Tf-1), *T. ferrooxidans* ATCC 19859 (Tf-2), and SM-1 to SM-8 were grown on sulfur or ferrous iron as described earlier. Collection of cells and activity determinations on sulfur or Fe^{2+} were also as described. Tf-1, Tf-2, SM-1, SM-3, SM-4, SM-5 and SM-8 strains used for sulfur growth were initially grown on Fe^{2+} in the M9K medium and were isolated as single colonies on the solid Fe^{2+} medium with Oxoid agar or agarose according to Harrison (1). Each isolated culture was adapted to growth on sulfur and was maintained in the Starkey's No. 1 medium on sulfur.

Ore

The sulfide ore samples used were obtained from the Hudson Bay Mining and Smelting Co. Ltd. at Flin Flon, Manitoba and contained chalcopyrite, sphalerite and pyrite. The No. 1 ore sample (ore-1) contained 4.9% Cu, 12.5% Zn, 30% Fe and 37.5% S. The No. 2 ore sample (ore-2) contained 3.7% Cu, 10.2% Zn, 29% Fe, and 34.6% S. The No. 3 ore sample (ore-3) contained 3.1% Cu, 9.1% Zn, 33.4% Fe and 37.8% S. The ore-1 was used in our previous shake flask experiments (2,3) and both the ore 1 and ore 2 were used in our previous column leaching studies (2,4).

Shake flask leaching experiment

All experiments were carried out in 250 mL Erlenmeyer flasks containing 90 mL of the HP medium with 10 g of -200 mesh ore and 10 mL of inoculum. The flasks were incubated at 25°C on a rotary shaker at 150 rpm. Samples of 5 mL were removed periodically and after filtration through a Whatman No. 1 filter paper and centrifugation at 12,000 x g for 10 min the clear supernatant solutions were analyzed for Cu, Zn, and Fe by atomic absorption spectrophotometry at the

Manitoba Provincial Soil Testing Laboratory.

In the experiments with Fe^{2+}-grown cells the remaining incubation mixture was filtered and centrifuged as above to obtain cells for activity determinations.

Column leaching experiment

The column leaching set-up was based on the method of McCready *et al.* (5) as described in (4). An acrylic plastic column (59 cm long x 7.5 cm I.D.) had a perforated acrylic base 3 cm from the bottom to hold the crushed ore and separate it from a bottom reservoir from which liquid was led by gravity to a culture reservoir (1 L Erlenmeyer flask).

The column was packed with a 3.4 kg, 50% w/w mixture of -3/4 in. size crushed ore and sand after agglomeration of the mixture by tumbling in a container with 4 mL of 95% sulfuric acid. The column was washed with several bed volumes of distilled water until the pH became approximately 2. The culture reservoir contained 500 mL of the HP medium plus 50 mL of inoculum which was circulated to the top of the column by a Gilson Minipuls 2 peristaltic pump at a rate of 2 mL/min. Aeration was provided by a glass sparger in the culture reservoir.

The system was run at 25°C. The sample of 5 mL was taken from the culture reservoir every 7-10 days for atomic absorption analysis after filtration and centrifugation. The pH was maintained at 2.2-2.5 with daily additions of 10 N H_2SO_4. At the end of each experiment lasting 34-40 days the culture flask content (450-500 mL) was replaced with 500 mL fresh HP medium (pH 2.3) for the next experiment. The flask content was filtered and cells were collelcted by centrifugation at 12,000 x g for activity determinations.

RESULTS

Shake flask leaching by sulfur-grown strains - No. 1 ore

Table 1 shows the results of leaching experiments with various strains with or without adaptation to Cu or ore. SM-6 and SM-7 were the best strains for leaching Cu and Zn especially after adaptation to ore. The results were similar to earlier ones (3). The pH of the uninoculated ore flask increased to 4.2 and slowly decreased to 3.8 over 2-3 weeks. Although all strains survived the incubation with the No. 1 ore for a month (they grew on sulfur afterwards), Tt and SM-5 did not improve much on the metal extraction after ore adaptation. Tf-1, Tf-2, SM-1, SM-3, SM-4 (Cu-adapted) and SM-8 partially adapted to ore and increased the metal extraction, although not to the level of SM-6 and SM-7. The final pH values generally agreed with the extent of metal extraction, the lower pH the better extraction. Generally also the adaptation to ore increased the metal extraction of strains. Unfortunately the supply of No. 1 ore was depleted and a further work was carried out with No. 3 ore-sample.

Shake flask leaching by sulfur-grown cells - No. 3 ore

Although the chemical compositions of No. 1 ore and No. 3 ore were similar, the incubation with No. 3 ore required the initial addition of 400 μL of 10 N H_2SO_4 to each flask of 100 mL HP medium to maintain the pH. Without the addition the pH increased quickly to 6 from 2.3 within 24 hours. With the acid addition the initial pH was 2, but increased to 4.2 in 4 days, then slowly to 5.2 by the 24 days of incubation without bacteria. With actively leaching bacteria the pH increased less in 4 days, then quickly dropped to below 2.5. These results are shown in Fig. 1. Washing in the HP medium for 3 hours did not eliminate the acid requirement of the No. 3 ore.

The results of leaching experiments by various strains are shown in Table 2. Tf-2, SM-6, SM-7 and SM-8 effectively leached metals and lowered the pH

without ore adaptation, although the ore adaptation further improved the performance. SM-1 leached metals well only after adaptation to ore and SM-4 required adaptation to Cu and ore before effective leaching. Tt, Tf-1, SM-3, SM-5, and SM-4 (without Cu adaptation) cells did not adapt to the No. 3 ore and died during the incubation. Effect of Cu adaptation was variable. It was definitely beneficial to SM-4, while not to SM-6. Although it did not show an improvement in the SM-7 results in Table 2 the Cu-adapted SM-7 leached Cu without a lag period, while the control SM-7 required several days of lag. Generally ore-adapted cells leached Cu with much shorter lag and lowered the pH faster than unadapted cells.

Shake flask leaching by iron-grown strains

Two samples of sulfide ores were used in these experiments. The ore-1 was the ore used in our previous work (2,3), but the supply was becoming depleted. The ore-2 had a similar composition but the leaching property was found to be quite different.

Table 3 shows the results of shake flask leaching experiments by various strains grown on Fe^{2+}. In addition to the solubilization of Cu, Zn and Fe and final pH the bacterial oxidation activity with Fe^{2+} or S^0 was studied after collection of cells from the filtered incubation mixtures. Since the cells adsorbed on the powdered ore were not collected the total activity would have been higher.

The results with the ore-1 were clear-cut with respect to the leaching abilities of various strains. The laboratory strains, Tf-1 and Tf-2, were not able to leach the metals beyond the control without bacterial inoculum. All the mine isolates leached ore effectively after adaptation to ore, although SM-3 results were variable. Without adaptation only SM-4 and SM-5 leached Cu and Zn consistently. SM-1, SM-2 and SM-8 gave variable results and SM-3 leached metals

only after ore adaptation. It is interesting that a culture derived from a single colony (SM-4 Pure) required ore adaptation before effective leaching. Cultures adapted to 50 mM Cu generally leached Cu better than non-adapted cultures, but the Cu-adapted Tf-1 and Tf-2 cultures were still unable to leach the No. 1 ore. Thus SM-4 and SM-5 are the most reliable and effective strains for Cu and Zn leaching, followed by SM-8, SM-1, SM-2 and SM-3, in that order. Tf-1 and Tf-2 are not suitable for metal extraction from this ore.

The Fe^{2+} and S^0 oxidation activities of collected free bacteria in Table 3 indicate that bacteria were definitely growing on the No. 1 ore when the effective leaching of Cu and Zn was achieved. With the 10% inoculum the activities without growth would have been 10% of those shown for Fe^{2+}-grown cultures. Some mine isolates grew much better on the ore than on Fe^{2+} as indicated by high activities, e.g. SM-1B-ore, SM-4A, SM-5A, SM-5B, SM-5B-ore, SM-8B, SM-8B-Cu, SM-8B-ore. Generally speaking the sulfur-oxidizing activity increased more than the Fe^{2+}-oxidizing activity when grown on ore (higher S^0/Fe^{2+} ratio), evidently due to the sulfide nature of substrate. The effective leaching of Cu and Zn was associated with the bacterial activity (Fe^{2+}) similar or higher than that of Fe^{2+}-grown culture, but a much higher activity did not necessarily lead to more leaching. Thus the leaching experiments with much better growth (or activity) mentioned above did not leach Cu and Zn any more than those with activity equal to the Fe^{2+}-grown cultures. It should be emphasized, however, that the activity determined was that of cells free from ore particles and not the total activity of cells including those adsorbed on the ore particles.

The results with No. 2 ore sample were quite different. It was necessary to add sulfuric acid to maintain the acidic medium pH similar to the No. 3 ore. Bacterial inoculation increased the Cu and Zn leaching but much less than with

No. 1 ore (less than doubling of Cu and around doubling of Zn) with or without Cu or ore adaptation. Cu adaptation generally increased the leaching slightly but ore adaptation often decreased it. The final pH was in most cases slightly lower than the uninoculated control (pH 2.8). This was in contrast to the results with No. 1 ore where the pH was much higher (above 4) than the control (pH 2.5) when there was no leaching beyond the control (Cu leaching was often lower than control). This result with No. 1 ore was probably due to bacterial adsorption to ore particles preventing the chemical leaching and acid production. There was little bacterial growth in these flasks. With the No. 2 ore, however, the bacterial growth was much better than in the Fe^{2+} medium or with the No. 1 ore in most cases, although the leaching of metals was much slower. Again the bacterial growth as measured by the oxidation activities had no relationship to the metal leaching rates and the sulfur-oxidizing activity to Fe^{2+}-oxidzing activity ratios were higher than Fe^{2+}-grown culture. The bacteria were perhaps growing on pyrite as substrate.

The different results between the No. 1 ore and No. 2 ore may lie in the electrochemical properties. The galvanic interaction which helps the leaching of chalcopyrite and sphalerite in the presence of pyrite was perhaps stronger in the No. 1 ore. The strain difference was much more evident with the No. 1 ore than with No. 2 ore.

Column leaching

Four small columns (7.5 x 59 cm) containing 1.7 kg No. 2 ore (-3/4") and 1.7 kg sand each as described in Materials and Methods were used for the column leaching study by four different strains. Strains selected were sulfur-grown SM-6 and SM-7 (both adapted to ore-1) and Fe^{2+}-grown SM-4 (adapted to ore-1) and Tf-2 (adapted to 50 mM $CuSO_4$). SM-6 and SM-7 were grown on sulfur and SM-4 and Tf-2 were grown on Fe^{2+} as inoculum without ore or $CuSO_4$.

The results are shown in Table 4. In the Experiment 1 all four columns required a daily addition of around 1 mL of 10 N H_2SO_4 (considerably more than our previous work, refs. 2,4) to maintain the pH below 2.5. Surprisingly all four strains leached Cu and Zn effectively (1.5-2 g Cu/L and 19-28 g Zn/L) and the columns were filled throughout with large quantities of ferric iron precipitates due to Fe leaching from the ore. The Cu% extraction rate was similar but the Zn% extraction was faster than our previous results (2,4) perhaps due to larger quantities of sulfuric acid used. The Cu/Zn extraction ratio was 0.15 for columns 1 and 2 and 0.22-0.23 for columns 3 and 4. The sulfur-grown inocula (columns 1 and 2) containing *T. thiooxidans* SM-6 and SM-7 produced unexpectedly cells oxidizing Fe^{2+} faster than S^0 after 34 days of growth on the No. 2 ore, although the cells collected from the iron-grown inocula (columns 3 and 4) containing SM-4 and Tf-2 oxidized Fe^{2+} much faster.

In Experiment 2 there was a drastic decrease in acid requirement and after Day 45 the pH remained below 2.4 without any acid addition probably due to faster production of sulfuric acid from sulfide or sulfur. Cu and Zn extraction rates decreased in all columns, but more so in columns 3 and 4 and Fe extraction completely stopped. The Cu/Zn extraction ratio was around 0.2 in all columns. Bacterial activity remained unchanged in columns 1 and 2, but the sulfur-oxidizing activity decreased in columns 3 and 4.

In Experiment 3 the acid requirement completely disappeared and the extraction of Cu increased in all 4 columns. There was no Fe extraction. The Cu/Zn extraction ratio increased to 0.4 to 0.7 (column 1). The bacterial activity (both Fe^{2+} and S^0) increased in columns 1 and 2, while it decreased in columns 3 and 4.

With the introduction of Fe^{2+}-grown SM-4 to columns 1 and 2 and S^0-grown SM-7 to columns 3 and 4 in Experiment 4 the metal extraction rates were little

affected, and the bacterial activity decreased. Acid addition was not required and Fe extraction was none.

In Experiment 5 the requirement for acid addition reappeared and the quantity of acid required was similar to Experiment 1. Both Cu and Zn extraction rates increased and Fe extraction became considerable (4-9 g/L). The Cu/Zn extraction ratio became close to 1 in all columns. The concentration of Cu reached 4 to 5 g/L and that of Zn 15 to 20 g/L. The bacterial activity increased in columns 3 and 4, but disappeared in column 1. Since the leaching in column 1 was similar to other columns the last result could have been due to adsorption of cells to ore surfaces. It should be emphasized that we could measure only the bacterial activity appearing in the liquid medium fraction, i.e. the activity of bacteria free in suspension, not adsorbed to the ore.

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Table 1. Shake flask leaching of No. 1 ore by sulfur-grown strains.

Bacterial strains	Adaptation	Metal extraction (%)			Final pH
		Cu	Zn	Fe	
None		0.1	10.2	0.6	3.8
Tt		1.2	9.1	1.8	3.6
Tt	Ore	1.6	11.8	1.5	3.5
Tf-1		0.2	11.6	0.0	4.0
Tf-1	Ore	2.5	17.0	2.5	2.9
Tf-2		2.1	12.6	2.3	2.5
Tf-2	Ore	3.3	20.1	3.6	2.3
SM-1		0.8	10.9	0.1	3.8
SM-1	Ore	2.6	16.8	2.6	2.9
SM-3		1.9	12.2	1.9	2.9
SM-3	Ore	2.8	17.6	0.3	2.7
SM-4		1.0	8.0	0.7	3.8
SM-4	Ore	1.7	11.7	1.7	3.4
SM-4	Cu	2.2	12.2	1.8	2.4
SM-4	Cu, Ore	4.5	16.2	2.5	2.3
SM-5		0.8	11.2	0.1	3.8
SM-5	Ore	1.1	10.3	0.2	3.6
SM-6		3.4	21.6	2.3	2.4
SM-6	Ore	10.1	73.9	24.7	2.1
SM-6	Cu	3.1	14.9	2.0	2.5
SM-6	Cu, Ore	4.9	24.6	3.3	2.3
SM-7		2.1	8.8	2.2	2.9
SM-7	Ore	15.2	97.2	27.4	2.1
SM-7	Cu	3.5	18.5	1.9	2.3
SM-7	Cu, Ore	6.7	31.8	3.4	2.2
SM-8		2.1	12.6	1.9	2.6
SM-8	Ore	2.6	16.8	3.0	2.5

Shake flask leaching (150 rpm) of -200 mesh No. 1 ore (4.9% Cu, 12.5% Zn, 30% Fe) 10 g in 100 mL HP medium for 21 days at 25°C with 10% inoculum of sulfur-grown stationary cultures on Starkey's No. 1 medium. Tf-1, Tf-2, SM-1, SM-3, SM-4, SM-5 and SM-8 strains were from pure cultures adapted to growth on sulfur from single colonies isolated on a solid Fe^{2+}-agar (Oxoid agar or agarose). Gradual adaptation to Cu was achieved to a final concentration of 50 mM $CuSO_4$ in the Starkey No. 1 medium and the culture was regrown in the same medium without $CuSO_4$ to obtain the inoculum. The ore-adapted inoculum was obtained from previous shake flask experiments. Adaptation to ore was repeated, then the culture was regrown in the Starkey No. 1 medium with sulfur to obtain the inoculum.

Table 2. Shake flask leaching of No. 3 ore by sulfur-grown stains.

Bacterial strains	Adaptation	Metal extraction (%)			Final pH
		Cu	Zn	Fe	
None		0.0	1.9	0.0	5.2
Tt		0.1	2.1	0.0	4.9*
Tf-1		0.0	15.1	0.0	4.8*
Tf-2		18.0	64.1	4.7	2.1
Tf-2	Ore	23.1	105.8	24.8	1.4
SM-1		0.4	21.8	0.0	4.8
SM-1	Ore	24.3	114.0	30.6	1.3
SM-3		0.0	12.7	0.1	4.4*
SM-4		0.4	22.8	0.0	4.7*
SM-4	Cu	6.0	24.5	2.6	2.1
SM-4	Cu, Ore	24.0	79.1	6.4	1.8
SM-5		3.2	21.0	0.0	4.8*
SM-6		6.4	36.7	3.2	2.1
SM-6	Ore	24.0	114.0	30.6	1.3
SM-6	Cu	2.0	23.7	0.0	4.6
SM-6	Cu, Ore	16.9	59.0	2.0	2.4
SM-7		11.5	43.2	3.4	2.2
SM-7	Ore	25.0	119.5	3.3	1.3
SM-7	Cu	7.3	43.5	3.3	2.1
SM-7	Cu, Ore	17.4	55.2	2.0	2.4
SM-8		12.8	49.4	3.9	2.0
SM-8	Ore	22.9	119.5	32.0	1.3

Shake flask leaching of -200 mesh No. 3 ore (3.1% Cu, 9.1% Zn, 33.4% Fe) at 25°C. The conditions were the same as in Table 1, except that No. 3 ore was used, 400 μL of 10 N H_2SO_4 was initially added to each flask and the incubation was for 24 days.

*No adaptation to the No. 3 ore. The cells were dead after the experiment (no growth on sulfur).

Table 3. Shake flask leaching experiments by Fe^{2+}-grown strains.

Bacterial strains	Substrate	Metal extraction (%)			Final pH	Bacterial activity** (nmol O_2/min/mL)		
		Cu	Zn	Fe		Fe^{2+}	S^0	S^0/Fe^{2+}
None	Ore-1	2.2	14.2	1.7	2.5			
	Ore-2	1.9	4.1	2.7	2.8			
Tf-1	Fe^{2+}					10.5	0.30	0.03
Tf-2	Fe^{2+}					12.2	0.55	0.05
SM-1	Fe^{2+}					14.3	0.13	0.01
SM-2	Fe^{2+}					2.6	0.03	0.01
SM-3	Fe^{2+}					3.2	0.04	0.01
SM-4	Fe^{2+}					4.7	0.22	0.05
SM-5	Fe^{2+}					4.5	0.06	0.01
SM-8	Fe^{2+}					5.2	0.15	0.03
Tf-1	Ore-1	1.1	13.6	0.0	4.3			
Tf-1-Cu	Ore-1	1.5	14.0	0.0	4.2			
Tf-2	Ore-1	1.7	19.6	0.0		1.0	0.3	0.30
Tf-2-Cu	Ore-1	1.4	13.9	0.0	4.3	0.3	0.05	0.17
SM-1A	Ore-1	1.5	17.0	0.0	4.3*			
SM-1B	Ore-1	8.2	97.6	24.4	1.7*			
SM-1B	Ore-1	1.0	11.1	0.0	4.3	2.7		
SM-1B-Cu	Ore-1	11.3	37.1	0.2	3.2	16.7	2.7	0.16
SM-1B-Ore	Ore-1	8.4	79.9	9.6	2.3	303.0	33.2	0.11
SM-2A	Ore-1	1.0	11.8	0.0	4.2*			
SM-2A	Ore-1	7.7	66.2	6.8	2.2	5.5	1.7	0.31
SM-3A	Ore-1	0.9	10.2	0.2	4.0	1.0		
SM-3B	Ore-1	1.3	15.4	0.0	4.0*			
SM-3B-Ore	Ore-1	8.5	74.1	10.8	1.7*			
SM-3B-Ore	Ore-1	3.0	15.8	0.0	3.3			
SM-4A	Ore-1	8.7	96.8	22.8	2.1	60.3	10.4	0.17
SM-4B	Ore-1	4.9	104.0	29.0	1.9	3.3	0.5	0.15
SM-4B-Cu	Ore-1	9.3	101.6	31.5	1.9	2.4	0.6	0.25
SM-4B-Ore	Ore-1	9.4	24.3	0.0	3.3			
SM-4B-Ore	Ore-1	8.1	81.6	14.1	1.6*			
SM-4Pure	Ore-1	1.4	16.3	0.0	*			
SM-4Pure-Ore	Ore-1	9.6	90.4	19.0	1.6*			
SM-5A	Ore-1	8.7	51.6	1.6	2.7	40.0		
SM-5B	Ore-1	9.1	33.1	0.7	2.9	24.4	3.0	0.12
SM-5B-Cu	Ore-1	15.7	23.7	0.0	3.7	3.5	0.4	0.11
SM-5B-Ore	Ore-1	4.8	62.2	0.8	2.4	22.2	2.3	0.10
SM-8A	Ore-1	1.4	15.9	0.0	4.1*			
SM-8B	Ore-1	7.0	93.6	27.6	1.6*			
SM-8B	Ore-1	11.8	84.3	12.0	2.2	115.7	17.6	0.15
SM-8B-Cu	Ore-1	10.7	73.7	7.8	2.3	156.5	15.6	0.10
SM-8B-Ore	Ore-1	10.3	83.2	10.2	2.2	128.7	14.2	0.11
Tf-1	Ore-2	2.2	7.3	2.4	2.7	6.4	1.5	0.23
Tf-1-Cu	Ore-2	3.5	8.8	4.8	2.3	50.4	9.6	0.19
Tf-2	Ore-2	2.5	7.7	3.6	2.8	4.1	0.6	0.15
Tf-2-Cu	Ore-2	3.2	9.0	3.9	2.6	3.0	3.1	1.03

...continued...

Table 3 (continued)

Bacterial strains	Substrate	Metal extraction (%)			Final pH	Bacterial activity (nmol O_2/min/mL)		
		Cu	Zn	Fe		Fe^{2+}	S^0	S^0/Fe^{2+}
SM-1B	Ore-2	2.9	9.0	4.8	2.4	68.3	1.2	0.02
SM-1B-Cu	Ore-2	3.1	8.3	4.7	2.4	45.1	6.1	0.14
SM-1B-Ore	Ore-2	2.7	10.1	7.1	2.4	150.4	16.9	0.11
SM-2A	Ore-2	2.0	7.0	2.0	2.8	75.0		
SM-3A	Ore-2	2.5	7.9	3.9	2.4	92.2	9.0	0.10
SM-3B-Ore	Ore-2	2.3	9.0	5.3	2.4	213.9	17.7	0.08
SM-4A	Ore-2	2.4	8.7	3.6	2.4	126.2	15.3	0.12
SM-4B	Ore-2	2.5	9.7	6.5	2.2	92.0	12.0	0.13
SM-4B-Cu	Ore-2	3.5	9.8	7.5	2.3	115.5	24.2	0.21
SM-4B-Ore	Ore-2	2.5	10.5	7.9	2.3	232.1	12.4	0.05
SM-5A	Ore-2	2.6	8.1	4.7	2.3	83.8	8.3	0.10
SM-5B	Ore-2	2.8	9.2	5.9	2.4	60.7	9.8	0.16
SM-5B-Cu	Ore-2	3.2	9.4	6.3	2.4	81.8	7.8	0.10
SM-5B-Ore	Ore-2	2.2	8.6	4.6	2.5	66.1	7.0	0.11
SM-8B	Ore-2	2.5	7.3	2.6	2.5	101.6	13.2	0.13
SM-8B-Cu	Ore-2	3.2	8.2	3.8	2.4	110.7	12.0	0.11
SM-8B-Ore	Ore-2	2.4	9.5	4.7	2.4	131.4	9.5	0.07

Shake flask leaching (150 rpm) of -200 mesh No. 1 ore (4.9% Cu, 12.5% Zn, 30% Fe) or No. 2 ore (3.7% Cu, 10.2% Zn, 29% Fe) 10 g in 100 mL HP medium for 21 days at 25°C with 10% inoculum of Fe^{2+}-grown bacterial strains indicated. Adaptation of strains to Cu (-Cu) was at 50 mM $CuSO_4$ in the Fe^{2+}-M9K medium and adaptation to ore (-Ore) was at 1 g No. 1 ore in 100 mL of the same medium followed by culturing in 10 g ore in 100 mL HP medium without Fe^{2+}. In both adaptation the above procedures were followed by growth in the Fe^{2+}-M9K medium without Cu or ore to obtain the inoculum for the leaching experiment. When Fe^{2+} was used as substrate the cells were grown for 2-3 days. A and B were different stock cultures for various strains. SM-1A, SM-2A, SM-4A and SM-5A were preserved in the presence of small quantity of ore. SM-4 Pure was a stock culture from an isolated colony on a solid Fe^{2+}-agar medium. It was necessary to add 250 μL 10 N H_2SO_4 to the No. 2 ore flasks before incoulation with bacteria and 25 μL again after 1 day.

*Experiments were for 19 days for metal extraction and the pH was determined after 60 days of shaking.

**Activity was determined in 0.1 M B-alanine buffer at pH 3.0 with 5 μmol Fe^{2+} or 24 mg sulfur in 1.2 mL (Oxygraph) at 25°C.

Table 4. Column leaching experiments of No. 2 ore.

Column	Bacterial strains	H_2SO_4 10 N (mL)	Metal extraction (%)			Bacterial activity* (nmol O_2/min/mL		
			Cu	Zn	Fe	Fe^{2+}	S^0	S^0/Fe^{2+}
Experiment 1 (Day 0-34)								
1	SM-6	33.1	1.1	7.5	0.18	0.25	0.01	0.04
2	SM-7	36.7	1.1	7.1	0.23	1.1	0.02	0.02
3	SM-4	32.8	1.4	6.0	0.33	9.9	0.65	0.07
4	Tf-2	39.9	1.1	5.1	0.25	17.8	0.67	0.04
Experiment 2 (Day 34-70)								
1	SM-6	5.6	0.54	2.3	-	0.44	0.01	0.02
2	SM-7	6.4	0.51	2.8	-	1.0	0.02	0.02
3	SM-4	5.2	0.30	1.4	-	4.7	0.13	0.03
4	Tf-2	5.4	0.47	2.3	-	12.5	0.09	0.01
Experiment 3 (Day 70-105)								
1	SM-6	0.0	0.78	1.1	-	1.1	0.04	0.04
2	SM-7	0.0	1.3	3.1	-	2.0	0.07	0.04
3	SM-4	0.0	1.1	2.1	-	1.1	0.06	0.05
4	Tf-2	0.0	1.0	2.2	-	3.5	0.11	0.03
Experiment 4 (Day 105-141)								
1	SM-6+SM-4	0.0	0.86	0.87	-	0.34	0.02	0.06
2	SM-7+SM-4	0.0	0.49**	1.2**	-	0.82	0.02	0.02
3	SM-4+SM-7	0.0	1.4	1.9	-	0.56	0.02	0.04
4	Tf-2+SM-7	0.0	1.2	2.0	-	1.2	0.05	0.04
Experiment 5 (Day 141-181)								
1	SM-6+SM-4	19.0	2.6	2.5	0.36	0.00	0.00	
2	SM-7+SM-4	30.0	2.4	3.0	0.61	0.75	0.04	0.05
3	SM-4+SM-7	31.0	2.6	3.5	0.82	1.0	0.05	0.05
4	Tf-2+SM-7	33.5	2.3	3.5	0.91	3.9	0.16	0.04

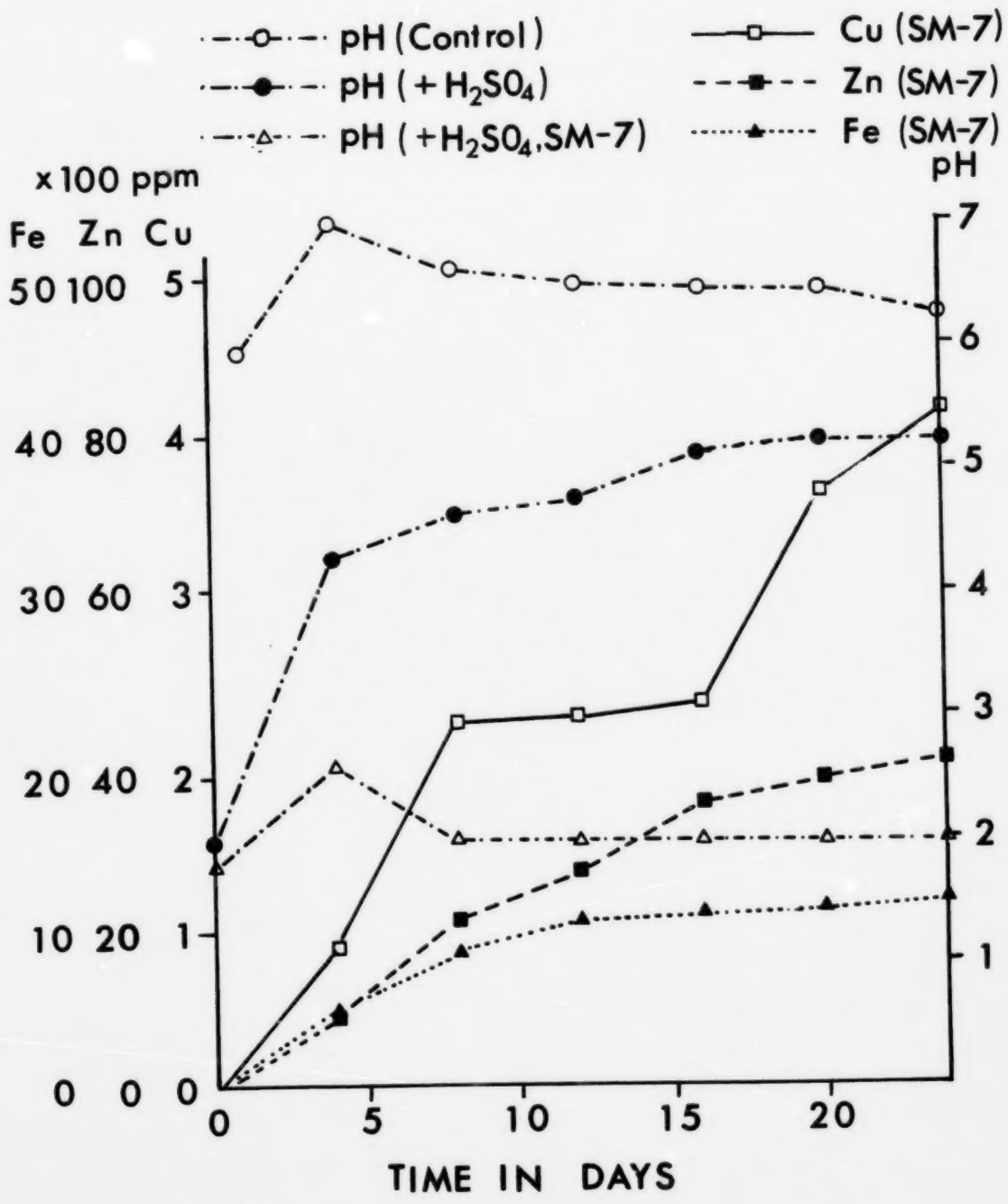
Four columns (1 to 4) each containing 1.7 kg ore-2 (-3/4") plus 1.7 kg sand were used for percolation leaching at 2 mL/min with 550 mL of bacterial cultures at 25°C as described in Materials and Methods. The pH was daily adjusted to 2.2-2.5 with 10 N H_2SO_4. At the end of each experiment 500 mL of fresh HP medium replaced the 450-500 mL old culture. In the Experiment 4 50 mL fresh culture was added to constitute the mixed culture columns.

*Bacterial activity was determined in the HP medium at pH 2.3 with 5 μmol Fe^{2+} or 24 mg sulfur in 1.2 mL (Oxygraph) at 25°C.

**The metal extraction for the first 15 days due to a spill.

Figure Legends

Figure 1. Shake flask leaching of No. 3 ore by Cu-adapted SM-7. The pH was recorded with or without 400 μL 10 N H_2SO_4 in control uninoculated flasks in the SM-7 flask (with the initial addition of H_2SO_4).


pH (Control)
pH (+H2SO4)
pH (+H2SO4,SM-7)
Cu (SM-7)
Zn (SM-7)
Fe (SM-7)
x100 ppm
Fe Zn Cu
50 100 5
40 80 4
30 60 3
20 40 2
10 20 1
0 0 0
pH
7
6
5
4
3
2
1
0
5
10
15
20
TIME IN DAYS

GENERAL CONCLUSION

GENERAL CONCLUSION

The response of various *Thiobacillus* strains on a number of parameters studied in this work is listed in Table 1.

The eight strains (SM-1 to 8) were isolated from water samples from the Flin Flon Mine of the Hudson Bay Mining and Smelting Co. Ltd., Manitoba by enrichment in the Starkey No. 1 S^0 medium or the M9K-Fe^{2+} medium as shown in Parameter 1 (P1). Three control ATCC strains (Tt, Tf-1 and Tf-2) and eight mine isolates were maintained either on S^0 (Tt, SM-6 and SM-7) or Fe^{2+} (Tf-1, Tf-2, SM-1 to 5, SM-8) as energy source (P2).

Adaptation of the Fe^{2+}-cultures to growth in the S^0-medium was relatively easy for Tf-1, Tf-2, SM-3, and SM-4, but was more difficult for SM-1, SM-5, and SM-8. SM-2 never grew in the S^0-medium (P3). All strains (except SM-2) grew well in the S^0-medium (P4).

Tolerance to 50 mM $CuSO_4$ during growth in the S^0-medium was higher with Tt, SM-6, SM-7, SM-3 and SM-4 than with Tf-1, Tf-2, SM-1, SM-5 and SM-8 (P5). Tolerance to 200 mM $ZnSO_4$ in the S^0-growth was higher with SM-6, SM-7, and SM-3 than with others (P6).

Adaptation to 50 and 100 mM $CuSO_4$ in the S^0-medium was good (P7) in all four strains (Tt, SM-6, SM-7 and SM-4) and all the adapted cultures required the presence of Cu both for good growth and good S^0-oxidizing activity (P8).

The sulfur-oxidizing activity of cells was higher in Tt, SM-6, SM-7, Tf-1, Tf-2, SM-1 than in SM-3, SM-4, SM-5 and SM-8 (P9). The activity was more resistant to inhibition by 167 mM $CuSO_4$ (P10) or 417 mM $ZnSO_4$ (P11) in SM-6, SM-7, SM-3, SM-4 and SM-8 than in Tt, Tf-1, Tf-2, SM-1 and SM-5.

Tt, SM-6 and SM-7 did not grow in the Fe^{2+}-medium and Tf-1, Tf-2, SM-3 and SM-4 grew faster than SM-1, SM-2, SM-5 and SM-8 (P12).

Tf-2, SM-1, SM-5 and SM-8 were more tolerant of 30-50 mM $CuSO_4$ during

growth on Fe^{2+} than Tf-1, SM-3 and SM-4 (P13). SM-2 failed to grow in the presence of 50 mM $CuSO_4$. Tf-1, Tf-2, SM-2, SM-3, SM-4, SM-5 and SM-8 strains were all tolerant of 150 mM $ZnSO_4$ in the Fe^{2+} medium (P14) and adapted well (P16). SM-1 failed to grow in the presence of 150 mM Zn. *T. ferrooxidans* strains except SM-2 adapted to 50 mM $CuSO_4$ (P15).

The Fe^{2+}-oxidizing activity was high in all *T. ferrooxidans* strains except SM-2 which had normally a lower activity (P17). All the mine isolates except SM-2 had higher affinities for Fe^{2+} as substrate (lower K_m) than laboratory strains, Tf-1 and Tf-2 (P18). The Fe^{2+}-oxidizing activity of many Cu-adapted mine isolates (SM-1, SM-3, SM-4 and SM-5) and Zn-adapted isolates (SM-3, SM-4 and SM-8) was activated by metals such as Cu^{2+}, Zn^{2+} or Fe^{3+} (P19,20).

All *T. thiooxidans* strains (Tt, SM-6 and SM-7) were tolerant of 5 μM Ag and of 5 μM Hg in the S^0-growth and metal adaptation (P21,22), while only Tf-2 and SM-8 were tolerant of both, SM-3 and SM-4 only of Ag, and Tf-1 of only Hg among *T. ferrooxidans*. Tolerance during the F^{2+}-growth and metal adaptation to 0.1 or 1 μM Ag or Hg (P23 and 24) was high for SM-4 (both Ag and Hg), Tf-1 and SM-3 (only Ag) and SM-8 (only Hg): medium for Tf-2, SM-2, and SM-5 (both Ag and Hg); SM-8 (only Ag); Tf-1, SM-1, and SM-3 (only Hg): and low for SM-1 (only Ag).

Tolerance to 100 μM Mo in the S^0-growth and metal adaptation was high in SM-6, SM-7, SM-4 and SM-8; medium in Tt, Tf-1, Tf-2, SM-1, and SM-5; and low in SM-3 (P25). Tolerance and adaptation to 100 μM and 1 mM Mo in the Fe^{2+}-growth were high in Tf-2, SM-3, SM-4 and SM-5; and medium in Tf-1, SM-1, SM-2, and SM-8 (P26). In the Fe^{2+} oxidation SM-3 was highly tolerant of Mo (K_i, 12 mM) and SM-5 extremely sensitive (K_i, 2-5 μM). Other strains were moderately tolerant (P27).

Tolerance to As (50 μM) in the S^0-growth was high in all the strains except SM-5 (P28) and tolerance to 1 mM As was high in all *T. ferrooxidans* strains

(P29).

The Cu and Zn leaching from the No. 1 ore by S^0-grown cells in the shake flask experiments was high in SM-6 and SM-7; medium in Tf-1, Tf-2, SM-1, SM-3, SM-4, and SM-8; and low in Tt and SM-5 (P30). The leaching from the No. 3 ore was high in SM-6, SM-7, Tf-2 and SM-8; medium in SM-1 and SM-4; and low in Tt, Tf-1, SM-3 and SM-5 (P31).

The leaching of Cu and Zn from the No. 1 ore by Fe^{2+}-grown cells in shaking flasks was high in SM-4 and SM-5; medium in SM-1, SM-2 and SM-8; low in SM-3; and very low in Tf-1 and Tf-2 (P32). The leaching from the No. 2 ore in shake flasks was medium in all strains (P33).

The column leaching of Cu and Zn from the No. 2 ore was good in all four strains tested (SM-6, SM-7, Tf-2 and SM-4) (P34).

The subtotal points for parameters relating to S^0-grown cells (P35) indicate that SM-6 and SM-7 have by far the highest points and SM-2 (no growth on S^0) and SM-5 have the lowest. SM-4 and SM-8 have high marks.

The subtotal points for parameters relating to Fe^{2+}-grown cells (P36) show that SM-4 has the highest mark, followed by SM-3, Tf-2, SM-5 and SM-8.

The total points for all parameters studied (P37) place SM-4 in the number 1 ranking followed by SM-3, SM-8 and Tf-2 among *T. ferrooxidans* strains. SM-6 and SM-7 are much better than Tt, the laboratory strain.

It is concluded that SM-4 is the best strain in all respects for bacterial leaching of metals from sulfide ores. Although SM-3 scored high in many parameters its metal-leaching ability was low and its potential is therefore questionable. Its high tolerance to Mo makes it a potential strain for an ore with high Mo content, perhaps in combination with SM-6 or SM-7 to complement its low score in S^0-cell parameters. Other high-scoring strains, SM-8 and Tf-2, perhaps after adaptation, have the potential to become effective

metal-leaching strains. Among *T. thiooxidans* strains both SM-6 and SM-7 scored very high in all respects including the metal-leaching ability and are good candidates for effective mineral leaching from sulfide ores. SM-5 scored high only when grown on Fe^{2+}. It has a potential for metal leaching perhaps in combination with SM-6 or SM-7. SM-1 was a poor performer either S^0-grown or Fe^{2+}-grown, and leached Cu and Zn only in some experiments. Tf-1 performed slightly better when Fe^{2+}-grown, but its metal leaching ability was even worse. Tt performed much below SM-6 and SM-7 with lower tolerance to metals and was unable to leach Cu or Zn from a sulfide ore. SM-2 with the lowest total score was unable to grow on S^0, sensitive to most metals except Zn and As, and leached Cu and Zn only sometimes.

It is recommended that other parameters not studied in this work, the ability to adsorb on solid surfaces (1,2) and the ability to oxidize sulfur or sulfite with ferric iron (3-5), also be considered in future in order to obtain the complete assessment of various strains for effective mineral leaching from sulfide ores. We are currently investigating these parameters of various strains.

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Table 1. Evaluation of various *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* strains on activity parameters.

Parameters	*T. Thiooxidans*			*T. ferrooxidans*							
	Tt	SM-6	SM-7	Tf-1	Tf-2	SM-1	SM-2	SM-3	SM-4	SM-5	SM-8
1. Isolation on		S^0	S^0			Fe^{2+}	Fe^{2+}	Fe^{2+}	Fe^{2+}	Fe^{2+}	S^0
2. Maintenance on	S^0	S^0	S^0	Fe^{2+}	Fe^{2+}	Fe^{2+}	Fe^{2+}	Fe^{2+}	Fe^{2+}	Fe^{2+}	Fe^{2+}
3. Adaptation from Fe^{2+} to S^0				+1	+1	0	-1	+1	+1	0	0
4. Growth on S^0	+1	+1	+1	+1	+1	+1	-1	+1	+1	+1	+1
5. Cu-tolerance (S^0-growth)	+1	+1	+1	0	0	0		+1	+1	0	0
6. Zn-tolerance (S^0-growth)	0	+1	+1	0	0	0		+1	0	0	0
7. Adaptation to Cu (S^0-growth)	+1	+1	+1						+1		
8. Cu-dependency after adapt.	+1	+1	+1						+1		
9. S^0-oxidation	+1	+1	+1	+1	+1	+1		0	0	0	0
10. Cu-tolerance (S^0-oxid)	0	+1	+1	0	0	0		+1	+1	0	+1
11. Zn-tolerance (S^0-oxid)	0	+1	+1	0	0	0		+1	+1	0	+1
12. Growth on Fe^{2+}	-1	-1	-1	+1	+1	0	0	+1	+1	0	0
13. Cu-tolerance (Fe^{2+}-growth)				0	+1	+1	-1	0	0	+1	+1
14. Zn-tolerance (Fe^{2+}-growth)				+1	+1	-1	+1	+1	+1	+1	+1
15. Adaptation to Cu (Fe^{2+}-growth)				+1	+1	+1	-1	+1	+1	+1	+1
16. Adaptation to Zn (Fe^{2+}-growth)				+1	+1	-1	+1	+1	+1	+1	+1
17. Fe^{2+}-oxidation				+1	+1	+1	0	+1	+1	+1	+1
18. Low K_m for Fe^{2+}				0	0	+1	0	+1	+1	+1	+1
19. Metal activation of Cu cells				0	0	+1		+1	+1	+1	0
20. Metal activation of Zn cells				0	0		0	+1	+1	0	+1
21. Ag-tolerance (S^0-growth)	+1	+1	+1	0	+1	0		+1	+1	0	+1
22. Hg-tolerance (S^0-growth)	+1	+1	+1	+1	+1	0		0	0	0	+1
23. Ag-tolerance (Fe^{2+}-growth)				+1	0	-1	0	+1	+1	0	0
24. Hg-tolerance (Fe^{2+}-growth)				0	0	0	0	0	+1	0	+1
25. Mo-tolerance (S^0-growth)	0	+1	+1	0	0	0		-1	+1	0	+1
26. Mo-tolerance (Fe^{2+}-growth)				0	+1	0	0	+1	+1	+1	0
27. Mo-tolerance (Fe^{2+}-oxid)				0	0	0	0	+1	0	-1	0
28. As-tolerance (S^0-growth)	+1	+1	+1	+1	+1	+1		+1	+1	0	+1
29. As-tolerance (Fe^{2+}-growth)				+1	+1	+1	+1	+1	+1	+1	+1
30. Cu,Zn from ore-1 (S^0-cells)	-1	+1	+1	0	0	0		0	0	-1	0
31. Cu,Zn from ore-3 (S^0-cells)	-1	+1	+1	-1	+1	0		-1	0	-1	+1
32. Cu,Zn from ore-1 (Fe^{2+}-cells)				-1	-1	0	0	-.5	+1	+1	0
33. Cu,Zn from ore-2 (Fe^{2+}-cells)				0	0	0	0	0	0	0	0
34. Cu,Zn from ore 2 (column)		+1	+1		+1				+1		
35. Subtotal points (S^0-grown cells)	6	15	15	3	6	3	-1	5	9	-1	8
36. Subtotal points (Fe^{2+}-grown cells)	-1	-1	-1	7	9	3	0	12.5	15	9	9
37. Total points	5	14	14	10	15	6	-1	17.5	24	8	17

+1: Good; 0: Average or Mediocre; -1: Poor or Negative